

## ORIGINAL ARTICLE

# Oncolytic DNX-2401 Virus for Pediatric Diffuse Intrinsic Pontine Glioma

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## ABSTRACT

**BACKGROUND**

Pediatric patients with diffuse intrinsic pontine glioma (DIPG) have a poor prognosis, with a median survival of less than 1 year. Oncolytic viral therapy has been evaluated in patients with pediatric gliomas elsewhere in the brain, but data regarding oncolytic viral therapy in patients with DIPG are lacking.

**METHODS**

We conducted a single-center, dose-escalation study of DNX-2401, an oncolytic adenovirus that selectively replicates in tumor cells, in patients with newly diagnosed DIPG. The patients received a single virus infusion through a catheter placed in the cerebellar peduncle, followed by radiotherapy. The primary objective was to assess the safety and adverse-event profile of DNX-2401. The secondary objectives were to evaluate the effect of DNX-2401 on overall survival and quality of life, to determine the percentage of patients who have an objective response, and to collect tumor-biopsy and peripheral-blood samples for correlative studies of the molecular features of DIPG and antitumor immune responses.

**RESULTS**

A total of 12 patients, 3 to 18 years of age, with newly diagnosed DIPG received  $1 \times 10^{10}$  (the first 4 patients) or  $5 \times 10^{10}$  (the subsequent 8 patients) viral particles of DNX-2401, and 11 received subsequent radiotherapy. Adverse events among the patients included headache, nausea, vomiting, and fatigue. Hemiparesis and tetraparesis developed in 1 patient each. Over a median follow-up of 17.8 months (range, 5.9 to 33.5), a reduction in tumor size, as assessed on magnetic resonance imaging, was reported in 9 patients, a partial response in 3 patients, and stable disease in 8 patients. The median survival was 17.8 months. Two patients were alive at the time of preparation of the current report, 1 of whom was free of tumor progression at 38 months. Examination of a tumor sample obtained during autopsy from 1 patient and peripheral-blood studies revealed alteration of the tumor microenvironment and T-cell repertoire.

**CONCLUSIONS**

Intratumoral infusion of oncolytic virus DNX-2401 followed by radiotherapy in pediatric patients with DIPG resulted in changes in T-cell activity and a reduction in or stabilization of tumor size in some patients but was associated with adverse events. (Funded by the European Research Council under the European Union's Horizon 2020 Research and Innovation Program and others; EudraCT number, 2016-001577-33; ClinicalTrials.gov number, NCT03178032.)

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**D**IFFUSE INTRINSIC PONTINE GLIOMA (DIPG), a condition that is often associated with mutations in histone protein H3 genes, is the leading cause of brain tumor–related death in childhood.<sup>1</sup> Treatment options are limited because of the location, infiltrative nature, and aggressive behavior of the tumor. Despite a transient benefit with standard radiotherapy, the prognosis is poor, with progressive neurologic deterioration, median survival of less than 1 year, and 2-year survival under 10%.<sup>2</sup>

The oncolytic virus HSV-G207 has been used in a phase 1 trial involving pediatric patients with high-grade glioma in the cerebral hemispheres.<sup>3</sup> The conditionally replicative oncolytic adenovirus DNX-2401 (tasadenoturev, or Delta-24-RGD [arginine–glycine–aspartic acid]) has been evaluated in a dose-escalation study involving adult patients with recurrent malignant glioma.<sup>4</sup> The findings from that study suggested that a single intratumoral dose of DNX-2401 elicited immune-cell infiltration in the tumor microenvironment, induced variable tumor responses, and possibly led to prolonged survival in 3 of 25 patients. There were also adverse events, most of which were of grade 3 or lower. In preclinical models, DNX-2401 was shown to have direct oncolytic activity against DIPG cells, elicited antitumor immune responses,<sup>5</sup> and showed a synergistic effect with radiotherapy.<sup>6</sup> We conducted a study to evaluate the feasibility, safety, and potential efficacy of intratumoral infusion of DNX-2401, followed by radiotherapy, in pediatric patients with newly diagnosed DIPG.

## METHODS

### OBJECTIVES

The primary objective of the study was to assess the safety and adverse-event profile of DNX-2401, administered in a single intratumoral infusion, in pediatric patients with newly diagnosed DIPG. The secondary objectives were to determine overall survival at 12 months and the percentage of patients with an objective response (a partial or complete response), to assess health-related quality of life, and to collect tumor-biopsy and peripheral-blood samples for correlative studies of the molecular features of DIPG and antitumor immune responses.

### PATIENTS

Patients were eligible if they were 1 to 18 years of age, had newly diagnosed DIPG (as confirmed on the basis of clinical and magnetic resonance imaging [MRI] features of an infiltrating tumor involving at least 50% of the pons with hypointense or isointense signal on T1-weighted images and hyperintense signal on T2-weighted images), and had a Lansky or Karnofsky performance-status score of 70 or higher. The Lansky performance-status score is designed for patients 1 to less than 16 years of age, and the Karnofsky performance-status score is designed for patients 16 years of age or older. The scores on both scales range from 0 to 100 in 10-point increments, with higher scores representing better well-being and performance status. Patients were required to have adequate hematologic, kidney, and liver function and no concurrent infectious, autoimmune, or immunodeficiency conditions, as indicated in the protocol, which is available with the full text of this article at NEJM.org. Patients with known Li–Fraumeni syndrome or germline deficit in the retinoblastoma pathway were not eligible for the study. Full eligibility criteria are provided in Table S1 in the Supplementary Appendix, available at NEJM.org.

Written informed consent was obtained from a parent or guardian of each patient; adolescents older than 12 years of age could sign the written informed consent form along with their parent or guardian. The study was approved by the institutional review board of the Clínica Universidad de Navarra and conducted under the authorization of the Spanish Agency of Medicines and Medical Devices.

### TREATMENT

This study used a dose-escalation and expansion design, with an initial planned cohort of three patients to be treated with a dose of  $1 \times 10^{10}$  viral particles of DNX-2401; subsequent patients would receive a dose of  $5 \times 10^{10}$  viral particles. If the initial dose led to unacceptable side effects, a lower dose of  $1 \times 10^9$  viral particles would be evaluated.

Preoperative MRI with fiducial markers was used for surgical planning in the first four patients. To improve the precision of the biopsy procedure in the remaining eight patients, preoperative imaging with fiducial markers was performed immediately in the intraoperative

MRI scanner at the beginning of the surgical procedure. Tumor biopsy with stereotactic guidance was performed through the middle cerebellar peduncle with the use of the BrainLab Vario-Guide frameless system.

During the surgical procedure and after the biopsy, a dual-channel cannula (Alyone MEMS Cannula, Alyone Lifesciences) was positioned 1 cm deeper than the biopsy site through the biopsy tract for intratumoral administration of DNX-2401. To confirm the site of virus infusion on the immediate postoperative MRI, 150 to 250  $\mu$ l of diluted gadolinium was infused through a lumen of the cannula at a rate of 0.9 ml per hour. Then, through a second independent lumen of the cannula, a single infusion of the planned dose of DNX-2401, suspended in 1 ml of 0.9% sodium chloride, was delivered at a rate of 0.9 ml per hour, for a duration of infusion of 67 minutes. After the virus infusion and surgical procedure, an immediate postoperative MRI without intravenous gadolinium was performed while the patient remained under general anesthesia to verify successful targeted delivery of the virus (Fig. S1).

This procedure was used in a previous case report involving Patient 2, who was included in the current study.<sup>7</sup> Pretreatment tumor-biopsy samples were histologically examined by a neuropathologist to confirm the diagnosis and archived for sequencing and storing at the University of Navarra Biobank (Pamplona, Spain). Treatment with standard, nonconformal radiotherapy was intended to begin 2 to 6 weeks after the administration of DNX-2401. The use of chemotherapy was permitted at the discretion of the referring physician.

#### **SURVEILLANCE AND FOLLOW-UP**

Evaluations at baseline included physical and neurologic examinations, assessment of performance status with the Lansky or Karnofsky performance-status score, assessment of health-related quality of life with the Pediatric Quality of Life Inventory 4.0 Generic Core Scales (in which four domains of functioning [physical, emotional, social, and school-related] are reverse-scored and transformed to a scale of 0 to 100, with higher scores indicating better quality of life),<sup>8</sup> MRI of the head, and blood tests. After DNX-2401 infusion, patients were evaluated for safety and tumor response every 4 weeks for

3 months. Evaluations included physical and neurologic examinations, assessment with the Lansky or Karnofsky performance-status score, health-related quality-of-life questionnaires, assessment of adverse events and ongoing medications, MRI of the head, and blood tests.

After the initial 3 months, patients continued to receive care at their respective referral hospitals. Remote follow-up was conducted by means of a telephone call or video conference every 6 months (or more frequently if necessary) for up to 2 years. Remote evaluations included clinical follow-up, a survey of adverse events, assessments of glucocorticoid use and health-related quality of life, and MRI analysis, if available.

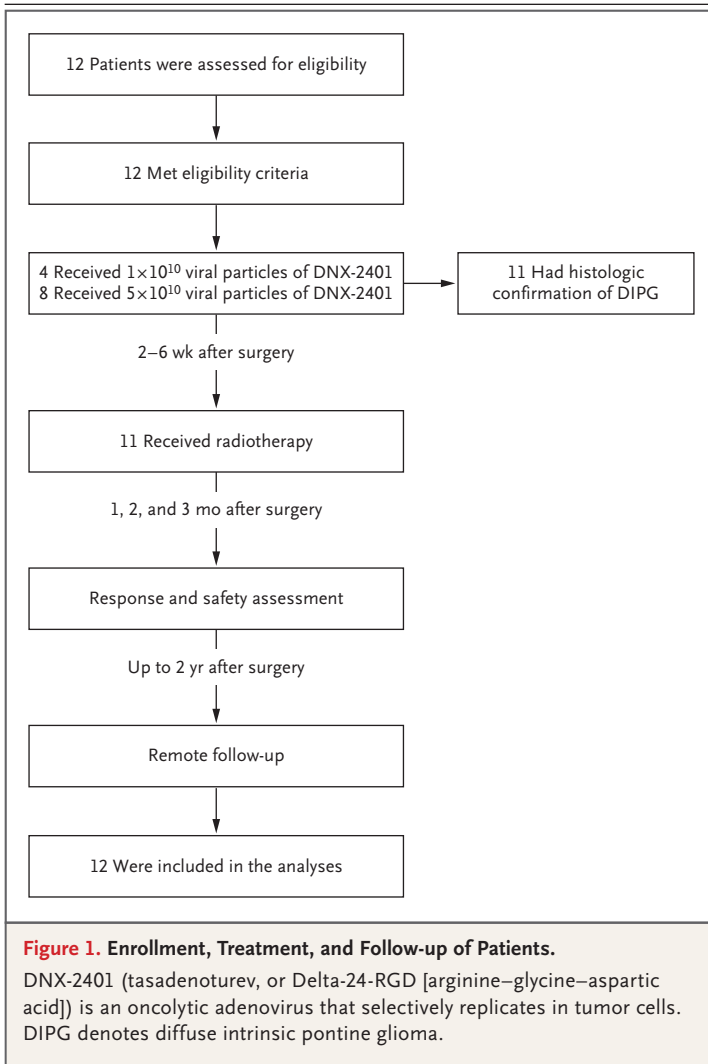
#### **SAFETY EVALUATION**

During the postoperative and follow-up periods, the patients were monitored for adverse events by the principal investigator or the pediatric neurologist at the study site; in the case of the latter, the report was later reviewed by the principal investigator. Adverse events were graded according to Common Terminology Criteria for Adverse Events, versions 4.03 to 5.0. A dose-limiting toxic effect was defined as any grade 3 or 4 adverse event that was at least possibly related to DNX-2401 within the first 2 months after administration. Because of the possibility of pseudoprogression (i.e., changes on clinical evaluation and imaging that mimic the presence of a progressive tumor but were due to therapy-related inflammation and edema) after both the study treatment and radiotherapy, patients with neurologic deterioration were allowed to be treated with glucocorticoids at the lowest dose needed.

#### **ASSESSMENT OF RESPONSE ON IMAGING**

MRI of the head was performed at screening, immediately after surgery, and during the outpatient visits that were conducted every 4 weeks for 3 months. Imaging sequences are described in the protocol. The results of additional MRI studies that were performed at the patient's referral centers were collected and used in the assessment of tumor response.

Tumor response was initially planned to be evaluated according to the adapted immunotherapy Response Assessment in Neuro-Oncology (iRANO) criteria.<sup>9</sup> However, after the publication of the Response Assessment in Pediatric Neuro-Oncology (RAPNO) criteria for DIPG<sup>10</sup> and be-



cause of the predominant nonenhancing character of DIPG, the protocol was amended to specify the RAPNO criteria for the evaluation of response. These criteria combine two-dimensional measures of the tumor on axial T2 or fluid-attenuated inversion recovery (FLAIR) sequences with clinical and functional outcomes and glucocorticoid dose. Progression on imaging was defined as an increase from baseline of 25% or more in the product of the maximal perpendicular diameters; an imaging response was defined as a reduction of 25% or more lasting at least 8 weeks. Response assessments were performed by the principal investigator, and imaging studies were also reviewed at a central location.

#### MOLECULAR AND IMMUNE ANALYSES

Tumor-biopsy samples were obtained during the surgical procedure, and peripheral-blood samples were collected preoperatively and postoperatively on the day of DNX-2401 administration and thereafter during outpatient visits at weeks 4, 8, and 12. Autopsy material was available from one patient. A detailed description of the methods used for molecular and immune studies is provided in the Supplementary Appendix.

#### STATISTICAL ANALYSIS

Adverse events were summarized according to frequency, grade, and relatedness to DNX-2401 as determined by the principal investigator of the study. The Kaplan–Meier method was used to analyze survival data. Overall survival was defined as the time from treatment with DNX-2401 to death or the time of the last follow-up contact. Progression-free survival was defined as the time from DNX-2401 administration to clinical or radiologic progression; data for the patients without progression were censored at the time of the last follow-up contact or at the initiation of new therapy in the absence of tumor progression. Statistical analyses were performed with the use of GraphPad Prism, version 9 (GraphPad Software), and Stata software, version 14 (StataCorp).

## RESULTS

#### PATIENT CHARACTERISTICS

From December 2017 through January 2020, a total of 12 patients (7 female and 5 male) with newly diagnosed DIPG were enrolled in the study (Fig. 1). The median age of the patients was 9 years (range, 3 to 18). The Lansky or Karnofsky performance-status score was 70 in 3 patients (25%), 80 in 5 patients (42%), 90 in 2 patients (17%), and 100 in 2 patients (17%). Patients had symptoms for a median of 32 days (range, 14 to 118) before enrollment. Dexamethasone (at a daily dose of 1 to 8 mg) was administered to 8 patients before DNX-2401 infusion. The baseline characteristics of the patients are provided in Table 1. Glucocorticoids were not used in any patient to treat changes related to pseudoprogression that were observed on imaging.

A histologic diagnosis of DIPG was confirmed in 11 of 12 patients. In the first treated

patient, the histologic results were inconclusive because the biopsy samples were obtained from the periphery of the lesion and did not contain tumor tissue for histologic and molecular characterization. This patient was included in the study analysis because the diagnosis relied on both clinical and imaging features; immediate postoperative MRI confirmed the site of intratumoral virus infusion. Oncogenic mutations were screened in the 11 available tumor samples (Table 1). A histone H3K27 mutation (H3K27M) was identified in 10 patients (83%), including *H3F3A* in 8 patients, *HIST1H3B* in 1 patient, and *HIST2H3C* in 1 patient. Mutations in *ACVR1* were identified in 4 patients (33%), and mutations in *TP53* were identified in 5 patients (45%); the mutations were mutually exclusive. The patient with H3K27M wild-type tumor had an *IDH1* p.(Arg132Gly) mutation. Detailed molecular features of tumor samples are summarized in Table S2.

#### TREATMENT

The first 4 patients received  $1 \times 10^{10}$  viral particles of DNX-2401, and the subsequent 8 patients received  $5 \times 10^{10}$  viral particles; a dose of  $1 \times 10^{10}$  viral particles was administered to the fourth patient because the safety review period for the first 3 patients had not yet been completed. MRI performed immediately after DNX-2401 infusion confirmed delivery to the pons and did not detect acute complications such as hemorrhage. The median length of hospital stay after intratumoral infusion of DNX-2401 and postoperative recovery was 2.3 days (range, 0.8 to 6.8).

Subsequent radiotherapy at a median dose of 54 Gy (range, 39.0 to 59.4) was administered to 11 of 12 patients. The parents of 1 patient declined radiotherapy and any further treatment. The median interval between intratumoral delivery of DNX-2401 and the start of radiotherapy was 17 days (range, 10 to 20). When there was tumor progression, patients received further treatment at the discretion of their oncologist. Further treatment included chemotherapy, reirradiation, antiangiogenic therapy, or investigational agents; a second intratumoral infusion of DNX-2401 was administered to 1 patient through compassionate use (Table S3). Three patients received additional treatment in the absence of tumor progression after the initial 3-month period after virus infusion.

**Table 1. Demographic and Clinical Characteristics of the Patients at Screening.**

Characteristic	Value
Median age (range) — yr	9 (3–18)
Sex — no. (%)	
Male	5 (41)
Female	7 (58)
Lansky or Karnofsky performance-status score — no. (%) <sup>*</sup>	
100	3 (25)
90	1 (8)
80	5 (41)
70	3 (25)
Patients receiving dexamethasone before surgery — no. (%)	8 (75)
Median score on the PedsQL 4.0 Generic Core Scales (range) <sup>†</sup>	80.4 (42.9–100)
Median symptom duration before enrollment (range) — days	32 (14–118)
Median baseline tumor size	
Longest diameter (range) — cm	4.6 (3.0–6.0)
Largest area (range) — cm <sup>2</sup>	17.6 (7.8–26.4)
Histone 3 mutation — no. (%)	
H3.1/H3.3 wild type	1 (8)
H3.1 K27M	1 (8)
H3.2 K27M	1 (8)
H3.3 K27M	8 (67)
Unknown	1 (8)
<i>TP53</i> mutation or wild type — no. (%)	
<i>TP53</i> mutation	5 (42)
<i>TP53</i> wild type	6 (50)
Unknown	1 (8)
Additional mutated genes — no. (%)	
<i>ACVR1</i>	4 (33)
<i>IDH1</i>	1 (8)
Other point mutations	5 (41)
Gene amplifications	2 (17)
No other detectable mutations	1 (8)
Unknown	1 (8)

<sup>\*</sup> The Lansky performance-status score is designed for patients 1 to less than 16 years of age, and the Karnofsky performance-status score is designed for patients 16 years of age or older. The scores on both scales range from 0 to 100 in 10-point increments, with higher scores representing better well-being and performance status.

<sup>†</sup> The Pediatric Quality of Life Inventory (PedsQL) 4.0 Generic Core Scales consist of four domains of functioning (physical, emotional, social, and school-related) that are reverse-scored and transformed to a scale of 0 to 100; higher scores indicate better quality of life.

**SAFETY**

The most frequent adverse events were headache, neurologic deterioration, and vomiting (in 9 patients each); fatigue (in 8 patients); and fever (in 6 patients) (Table 2). Three serious adverse events were reported. A transitory grade 3 hemiparesis related to the biopsy procedure developed in 1 patient (Patient 7) and lessened in severity to mild hemiparesis after 12 weeks. The preoperative MRI tractography for this patient showed motor tracts within the biopsy trajectories; the configuration of the tumor obligated the use of this trajectory, since different approaches were not available in this patient. Another patient (Patient 11) had grade 3 neurologic deterioration of increased bilateral oculomotor paresis and tetraparesis, which started after hospital discharge and worsened during radiotherapy. A third patient (Patient 2) was hospitalized for grade 1 abdominal pain 2 months after virus infusion; the pain resolved spontaneously, and ancillary tests did not show a cause of the symptoms. No grade 4 or 5 adverse events were observed. Among the 12 patients, 9 had at least one adverse event that was deemed by the principal investigator to be possibly related to intratumoral delivery of DNX-2401. Most of these events were of grade 1 (14 of 19 events) or grade 2 (4 of 19 events), with only 1 event of grade 3 (Tables S4 and S5). Before the occurrence of tumor progression on imaging, the delivery of DNX-2401 was not associated with a decline in quality of life or performance status (Fig. S2).

**CLINICAL AND IMAGING OUTCOMES**

Reductions in tumor cross-sectional areas were observed in 9 of 12 patients (75%) (Figs. S3 and S4) and were not discernibly related to the dose of DNX-2401. A total of 3 patients (25%) had a partial response (defined as a reduction of  $\geq 25\%$  in the cross-sectional area lasting  $\geq 8$  weeks according to the RAPNO criteria), as determined by the investigator, with durations of 3.5, 7.6, and 10.3 months. Two additional patients had a reduction in tumor size of more than 25% on follow-up imaging, but these reductions were not considered to be a partial response because the reductions were not maintained 8 weeks later. A best response of stable disease was observed in 8 patients (67%), including the 2 patients who had reductions in tumor size of more

than 25% that were not considered to be partial responses; thus, disease control (defined as a complete response, partial response, or stable disease) was achieved in 11 of 12 patients (92%) during a median follow-up of 16.6 weeks (range, 4.1 to 132.0). A best response of progressive disease was observed in 1 patient, in whom the tumor area increased from 17.6 cm<sup>2</sup> at baseline to 22.5 cm<sup>2</sup> at 2 months after surgery (an increase of 28.4%).

Establishment of the time of progression was difficult, given the potential for pseudoprogression after viral treatment. The median progression-free survival was 10.7 months (Fig. S5A). Data from 3 patients (Patients 1, 7, and 9) were censored at the start of new therapy in the absence of tumor progression. At the time of preparation of this report, Patient 8 remained free of progression 38 months after DNX-2401 administration.

The median overall survival was 17.8 months, with 3 patients remaining alive at 19.6, 31.4, and 33.5 months after DNX-2401 administration at the date of data cutoff for the analysis (Fig. 2); 2 patients were alive at the time of preparation of the current report. No substantial differences in overall survival were observed between the two DNX-2401 dose-level groups, nor in the analysis based on the presence or absence of TP53 variants (Fig. S5B and S5C). A total of 9 of 12 patients (75%) were alive at 12 months; 6 (50%) were alive at 18 months, and 3 (25%) were alive at 24 months. Tumor progression was the primary cause of death in all cases. The median follow-up was 17.8 months (range, 5.9 to 33.5). The clinical outcomes of the patients, along with their tumor mutational status and relevant clinical information, are summarized in Table S6.

**TOTAL IGG AND NEUTRALIZING ANTIBODIES**

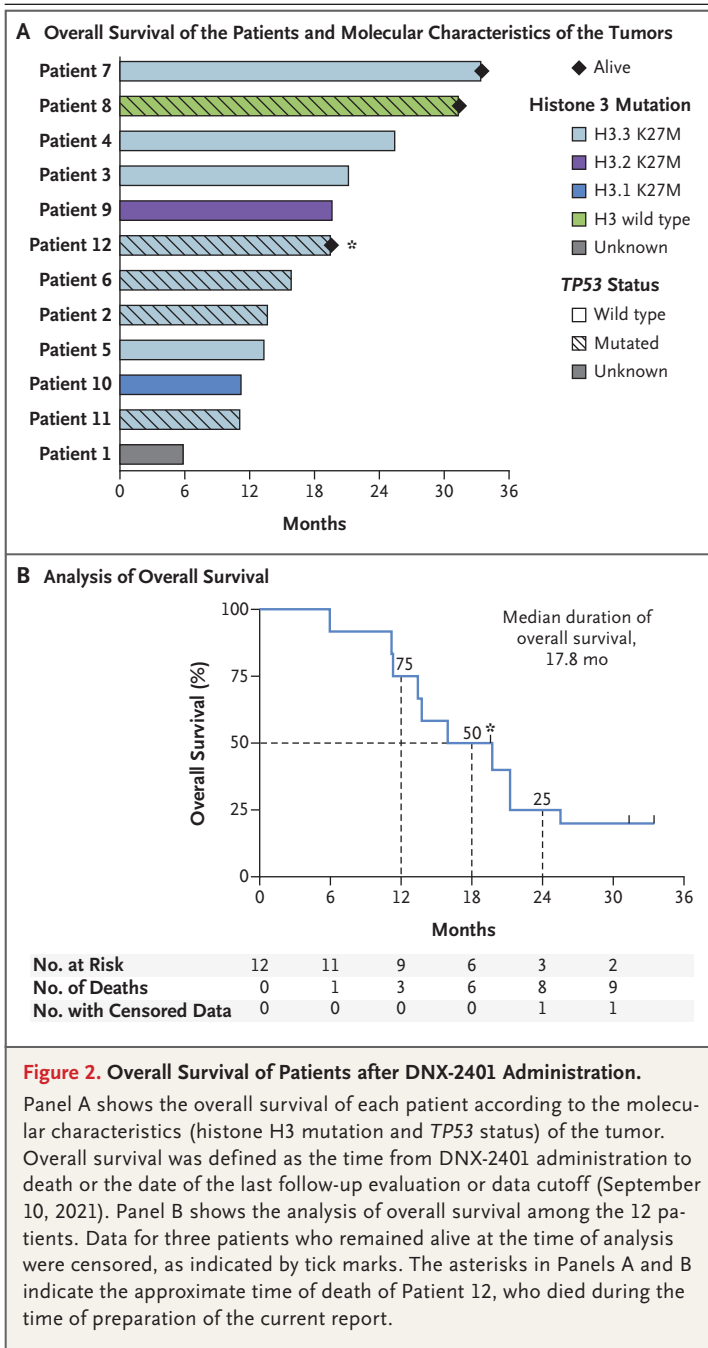
All the patients were seropositive for IgG antibodies against adenoviral hexon protein before treatment with DNX-2401, and the serum titers increased further after treatment (Fig. S6A). A total of six patients (50%) were positive for anti-adenoviral neutralizing antibodies before surgery, and the titers were increased 1 month after virus infusion (Fig. S6B). Seroconversion occurred in another five patients after infusion, whereas the titer of neutralizing antibodies in one patient remained below the threshold of detection. The median overall survival among the patients with

**Table 2. Adverse Events Recorded in the Period between DNX-2401 Administration and Tumor Progression.\***

Event	Grade 1 Events	Grade 2 Events	Grade 3 Events	Total Events	Patients with Event
					<i>number</i> <i>no. (%)</i>
Endocrine disorder: cushingoid	4	0	0	4	4 (33)
Gastrointestinal disorder					
Abdominal pain	4	0	0	4	3 (25)
Constipation	2	2	0	4	2 (17)
Dysphagia	2	0	0	2	2 (17)
Nausea	4	3	0	7	5 (42)
Vomiting	10	4	0	14	9 (75)
General disorder or administration-site condition					
Fatigue	12	0	0	12	8 (67)
Fever	7	2	0	9	6 (50)
Infection or infestation					
Thrush	2	0	0	2	1 (8)
Upper respiratory tract infection	2	1	0	3	3 (25)
Metabolism or nutrition disorder					
Hyperphagia	2	0	0	2	2 (17)
Anorexia	2	0	0	2	2 (17)
Musculoskeletal or connective-tissue disorder: neck pain					
Nervous system disorder					
Dizziness	2	2	0	4	4 (33)
Somnolence	2	2	0	4	3 (25)
Dysarthria	3	0	0	3	3 (25)
Hydrocephalus	2	0	0	2	2 (17)
Trigeminal nerve disorder	6	0	0	6	5 (42)
Facial nerve disorder	1	0	0	1	1 (8)
Headache	5	6	1	12	9 (75)
Muscle weakness, one-sided	0	0	2	2	2 (17)
Neurologic deterioration	6	3	1	10	9 (75)
Nystagmus	0	1	0	1	1 (8)
Ocular motor cranial nerve disorder	3	2	0	5	5 (42)
Psychiatric disorder					
Insomnia	6	0	0	6	4 (33)
Irritability	3	0	0	3	3 (25)
Renal or urinary disorder: dysuria					
Skin or subcutaneous tissue disorder					
Alopecia	3	0	0	3	3 (25)
Skin pain	2	0	0	2	2 (17)
Other†	15	6	0	21	9 (75)

\* Adverse events, except for “other,” are shown regardless of their causal relationship to the investigational drug and were graded according to Common Terminology Criteria for Adverse Events, versions 4.03 to 5.0.

† “Other” includes all adverse events that were reported only once and deemed by the principal investigator to be unrelated to DNX-2401.



**Figure 3 (facing page). Immunophenotype of Patient 9.**

Panel A shows representative immunofluorescence images of tumor samples from Patient 9. Immune-cell infiltration was observed in the samples obtained at the time of diagnosis (top), relapse (middle), and autopsy (bottom). Panel B shows the quantification of immune-cell populations in the tumor samples from Patient 9 at the time of diagnosis, relapse, and autopsy. Panel C shows cell-cluster maps obtained by means of single-cell transcriptome sequencing (scRNA-seq) analyses of samples obtained at the time of diagnosis, relapse, and autopsy. Panel D shows the frequency of different cell-subset populations. Panel E shows gene-ontology analysis of the tumor-infiltrating macrophages (TAMs) after DNX-2401 administration. Panel F shows the expression of marker genes from cytotoxic, effector memory, and regulatory tumor-infiltrating lymphocytes (TILs).

(Fig. S7A); myeloid cells (CD11b+) were the most abundant immune-cell population. Tumor samples obtained at the time of recurrence and at autopsy were available for one patient (Patient 9); on multiplexed immunofluorescence, the samples showed reshaping of tumor microenvironment at both time frames (Fig. 3A). Increased numbers of CD8+ and CD4+ T-cell infiltrates were observed at the time of relapse in this patient, as well as an absence of FoxP3+ regulatory T cells and a reduced number of myeloid cells (Fig. 3B). Reduced numbers of CD8+ and CD4+ T cells and an increased number of immunosuppressive M2 macrophages (CD163+) were also observed.

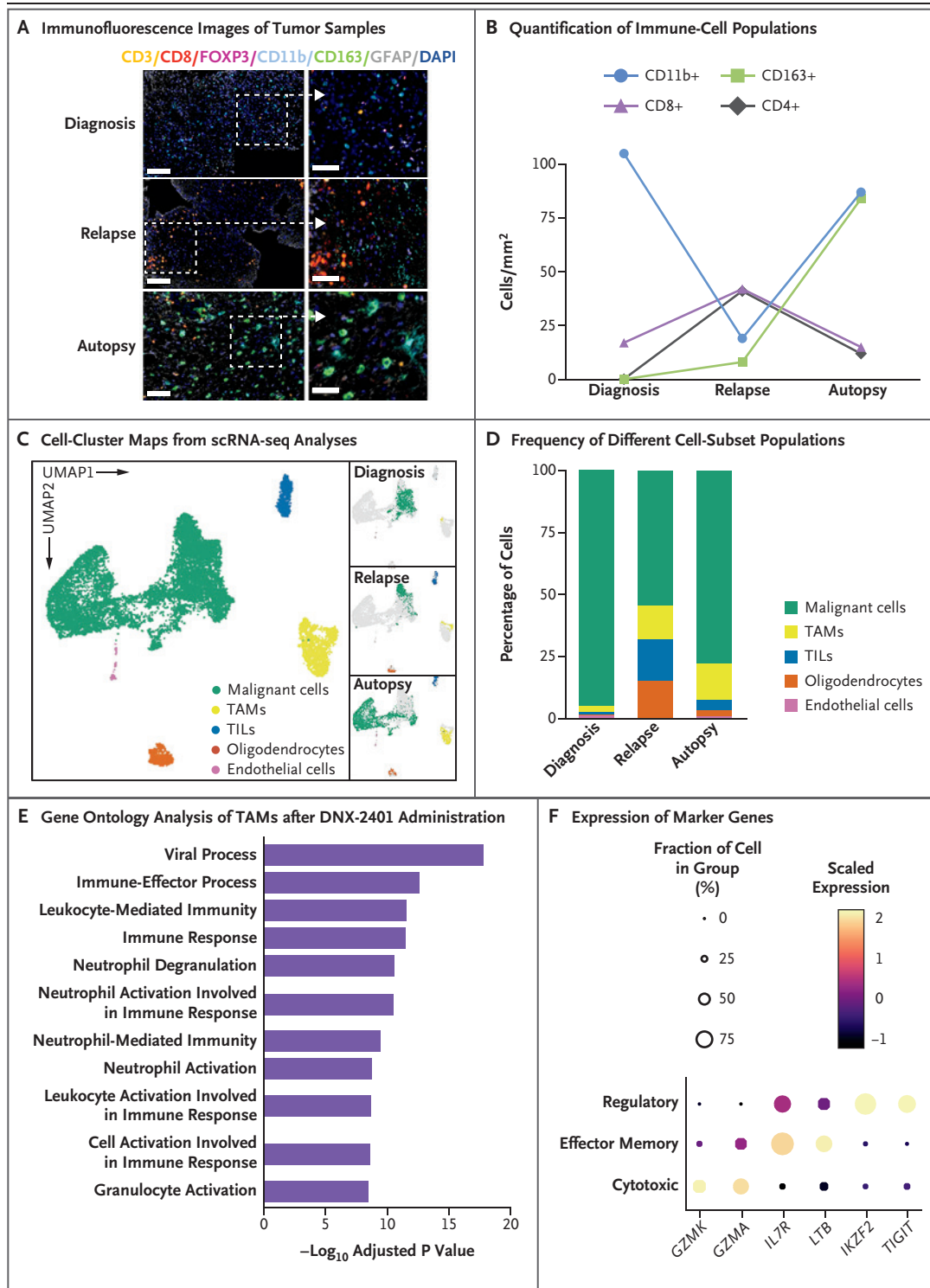
Single-cell RNA sequencing in the initial diagnostic biopsy sample from Patient 9 showed five different cell population clusters, including malignant cells (malignant status was assigned on the basis of inferred copy-number variation), and subsequent sequencing showed substantial differences among pretreatment, relapse, and autopsy samples (Fig. 3C and Fig. S7B and S7C). The pretreatment biopsy sample showed low percentages of tumor-infiltrating lymphocytes and macrophages, which were more prominent in the sample obtained at the time of relapse (Fig. 3D). After DNX-2401 administration, tumor-infiltrating macrophages showed an up-regulation of pathways associated with viral processes and enhanced immune response (Fig. 3E and 3F). At the time of relapse, tumor-infiltrating macrophages displayed overexpression of proinflammatory cytokines such as *CCL4* and *CCL3* (Fig. S7D). The chemoattractant signaling from the tumor-infiltrating macrophages was markedly as-

lower antibody titers after DNX-2401 infusion was 21.2 months, as compared with 12.5 months among those with higher titers (Fig. S6C).

**IMMUNOPHENOTYPING OF TUMOR TISSUE**

Immunophenotyping of tumor samples acquired before DNX-2401 administration revealed scarce immune-cell infiltration by CD8+ and CD4+ T cells (including FoxP3+ regulatory T cells)





sociated with the increase in tumor-infiltrating lymphocytes composed of cytotoxic, effector memory, and regulatory T cells (Fig. 3F and Fig. S7E). The relative increase in the percentage of myeloid cells in the sample obtained at autopsy featured

expression of M2-like marker genes (e.g., *CD163* and *TGFBI*), a finding consistent with those from the multiplexed immunofluorescence and could be linked to the decrease in the level of tumor-infiltrating lymphocytes.

### IMMUNE REPERTOIRE IN BLOOD MONONUCLEAR CELLS AFTER TREATMENT

The immune repertoire analysis of peripheral-blood mononuclear cells revealed a decrease in T-cell receptor diversity, as measured by the Shannon Diversity index, 2 months after receiving the virus infusion, as compared with that before treatment. It maintained the same tendency during the following month (Fig. S8A). In line with this trend, the higher T-cell receptor clonality during the following month was positively correlated with progression-free survival (Fig. S8B). In addition, at this time point, an increase in the frequency of overlapping T-cell receptors, as compared with that at baseline, was observed. Such an increase suggests that DNX-2401 had induced expansion of previously present clonotypes (Fig. S8C).

### DISCUSSION

Clinical trials of intratumoral oncolytic virotherapy in adult and pediatric patients with supratentorial high-grade gliomas have shown apparent responses and improved survival outcomes in some patients, as compared with historical controls.<sup>3,4,11</sup> Data from trials of direct infusion of an oncolytic virus in children with DIPG are lacking owing to the difficulty of reaching the location of the brain-stem tumor and the concern over procedure-associated complications and virotherapy-related inflammation in the pons that contains many ascending and descending fiber tracts and nuclei in a small area.

In the current study, the median overall survival was 17.8 months, and the overall survival rate at 18 months was 50%. Of the 12 patients included in our study, 9 lived more than 12 months after DNX-2401 administration, including 3 who lived longer than 24 months. The patient who received no further antitumor treatment after virus delivery survived for 13.4 months after DNX-2401 administration. Of the 2 patients with the longest survival time, 1 had a tumor that was H3 wild type and *IDH1* (p.Arg132Gly) mutated. This molecular profile has been associated with a better outcome than in the tumors without these changes.<sup>12-14</sup> In previous studies involving patients with DIPG, the median overall survival with radiation therapy alone was reported to be generally between 8 and 12 months,<sup>15,16</sup> with minimal benefit from the addition of nimotuzumab,<sup>17</sup> everolimus, dasatinib, or erlotinib to

radiotherapy or the use of reirradiation at recurrence.<sup>18</sup> However, the small sample size in our study and the lack of a randomized design preclude any conclusion regarding survival. There is preclinical evidence of direct oncolytic activity of our treatment model and suggestive evidence that this model was operative in our patients, but we have limited information to address the relative contributions of the change in inflammatory response in the tumor and viral oncolytic activity.

Given the potential for pseudoprogression after both virotherapy and radiotherapy, the duration of progression-free survival was difficult to determine with certainty. However, this phenomenon was not detected on follow-up MRI, which included only T2-weighted FLAIR sequences owing to the typically diffuse, nonenhancing pattern of these tumors. Accordingly, no additional glucocorticoids were administered specifically for the treatment of pseudoprogression. Another challenge encountered in this study was the imaging evaluation of response to treatment. The recently described RAPNO criteria<sup>10</sup> were introduced in the study despite an original plan to use iRANO criteria because of their specific suitability for response assessment in patients with DIPG.

The results of the current study suggest that intratumoral infusion of DNX-2401 before radiation therapy is feasible in children with DIPG, providing a rationale for the conduct of a larger trial.

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#### APPENDIX

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