BMC Cancer

RESEARCH

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The safety and feasibility of multiple intrathecal injections of allogenic NK cells in pediatrics with refractory/recurrent brain tumors

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Abstract

Background Pediatric glioma is a rare condition that can lead to significant mortality and morbidity due to its high recurrence rate. This study is a phase I nonrandomized clinical trial that was conducted to assess the safety, feasibility, and potential efficacy of the intrathecal (IT) injection of multiple doses of allogenic NK cells in pediatric patients with refractory/recurrent gliomas.

Methods Allogeneic NK cells were isolated from random healthy unrelated donors via positive selection of CD56 + cells. Nine patients were selected according to the inclusion criteria and received weekly doses of up to 10 doses of 5×10^7 NK cells/injection. Adverse events grading was done based on Common Terminology Criteria for Adverse Events (CTCAE) Check lists. The size of the tumor, degree of spinal spreading and duration of relapse during 18 month followup were considered components of efficacy. Additionally, six patients who received conventional treatment were selected retrospectively.

Results Multiple intrathecal injections of allogeneic NK cells in pediatric gliomas were safe, without any serious adverse events (SAEs). The most prevalent AEs were headache [29% (17% grade 1 and 13% grade 2)], fever and chills [21% (17% grade 1 and 4% grade 2)], vomiting [13% grade 2], and back pain [12% (4% grade 1 and 8% grade 2)]. 18 months of follow-up, among the five patients in the intervention group who were still alive (August 7, 2024), three exhibited stable disease (SD), one had progressive disease (PD), and one experienced a partial response (PR) with a reduction in tumor size. Among the four deceased patients, two died due to tumor progression, and two died due to infections. In the retrospective control group, five out of six patients developed PD and leptomeningeal spread (LMS), four of whom died, and one patient showed radiological evidence of a complete response (CR). Cerebrospinal

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fluid (CSF) analysis revealed increases in the percentages of NK and T cells and significant reductions in the levels of IFN-γ and TNF-α.

Conclusions Multiple intrathecal injections of allogeneic NK cells are safe and feasible in pediatric patients with refractory/recurrent gliomas. Although we reported a reduction in recurrence episodes and an increase in overall survival, further studies with extended follow-up periods and appropriate control groups are necessary to assess the efficacy of NK cell therapy in these patients.

Trial registration The trial was registered on the Iranian Registry of Clinical Trials (IRCT20170122032121N6), Date 2021–11-19.

Keywords NK cell therapy, Refractory/recurrent glioma, Immunotherapy, Intrathecal injections

Introduction

Gliomas are the most common malignant CNS tumors in pediatric patients, with a high rate of mortality and low survival. They are classified on the basis of their histopathological and molecular features, and the most common types include astrocytomas, oligodendrogliomas, and ependymomas. The 2-year overall survival has been reported to be less than 50% in most surveys, and the only conventional treatments that have been shown to significantly increase survival rates are surgical resection and temozolomide-based chemotherapy, both of which have small effect sizes [1, 2]. Recurrence is a major challenge, with a survival rate of less than 10% despite various salvage therapies. Reradiation and reoperation are two inevitable treatment options [3, 4]. These limitations emphasize the urgent need for novel therapeutic approaches. It is essential to consider that locoregional techniques are necessary to overcome the challenges posed by the blood–brain barrier (BBB).

Natural killer (NK) cell therapy has emerged as a promising immunotherapeutic strategy for treating various malignancies, including gliomas [5], and for targeting cancer stem cells [6, 7]. Several clinical studies have shown that autologous or allogenic NK cell administration through systemic or intraventricular injection is safe but results in partial tumor inhibition [8]. Our preclinical work demonstrated the cytotoxic capacity of purified allogenic NK cells against a GBM cell line as well as patient-derived glial cells in vitro [9]. In a separate preclinical model, we revealed that allogenic NK cells could efficiently target tumor cells in a rat xenograft model [10]. In our previous clinical study, we reported that local administration of haploidentical NK cells increased the disease control rate by 33%, improved overall survival, and prevented tumor regrowth at the site of administration (tumor recurrence was observed in other sites of the CNS) but did not inhibit leptomeningeal spread (LMS) [11, 12]. The main reason for the inefficacy may be inadequate NK cell injection and local administration, which limits the circulation of NK cells in the whole CSF. Therefore, the administration of multiple doses of NK cells through the CSF and circulation throughout the entire central nervous system (CNS) may be more effective in inhibiting tumor regrowth and limiting LMS. To improve the efficacy of NK cells and eliminate these obstacles, we designed the current phase I trial. In the present study, 10 repeated intrathecal injections were administered to patients, and the safety of this intervention was assessed as the primary outcome. Furthermore, by incorporating retrospective controls, we aimed to better understand the differences in the clinical and radiological responses between the intervention and control groups. Moreover, through CSF analysis, including hematology, biochemistry, immunophenotyping of infiltrated cells, and cytokine assays, we are looking for immune responses in NK cell-treated patients and seeking a better understanding of the mechanism of the treatment response [12].

Methods

Study design

This study was an open-label, nonrandomized, nonparallel phase I clinical trial that was registered in the Iranian Registry of Clinical Trials (IRCT20170122032121 N6) on 2021–11–19.

The ethics code was obtained from the Royan Ethics Committee before patient enrollment (IR.ACECR.ROYAN.REC.1400.077) 2021–09–21. Patient enrollment was performed in consecutive order on a timeline from the beginning of the project. The recruitment and injection of NK cells took place at Rasoul Akram Hospital, which is affiliated with the Iran University of Medical Sciences in Tehran, Iran (between November 2021 and April 2022). Six age-matched patients who had received standard treatment at the same hospital were retrospectively selected as historical controls. Each patient was followed for 18 months after the final injection. The trial was designed and conducted in accordance with the Declaration of Helsinki and Ethical Guidelines for Clinical Research. The study was approved by the Royan Institutional Scientific Review Board. All pediatric patients were enrolled after signing a written informed consent form signed by their legal guardians. The Royan Institute Data

Safety Monitoring Board (Royan-DSMB) monitored possible adverse effects (AEs) and reported updates of the clinical trial in a timely manner.

Nine patients were selected and administered two cycles of activate allogenic NK cells. Each cycle involved five weekly intrathecal injections (4–5 $\times 10^7$cells/injection) through lumbar punctures. Two separate injection protocols were developed on the basis of whether the patient was receiving chemotherapy or radiation at the time of study enrollment.

The treatment protocol for patients undergoing chemotherapy involved administering NK cells on a weekly basis up to five injection, timed with their chemotherapy sessions. The initial dose was given one week after the final chemotherapy session to minimize chemotherapy's impact on NK cell survival. For patients who were undergoing radiotherapy, the first NK cell cycle also was began one week post-radiotherapy, with weekly injections thereafter up to five injections. An MRI scan was performed one month after the fifth injection to check for any new neurological lesions or disease progression. If new lesions or disease progression were detected, NK cell injections were finished. If no progression was observed, a second cycle of NK cell injections was administered between chemotherapy courses, spaced a week apart. To screened adverse events, patients were hospitalized and closely observed for 24 h following each NK cell injection. Figure 1 illustrates the entire treatment protocol.

The secondary outcomes primarily focused on response assessment, which included changes in tumor size, and clinical status, patient survival, and CSF analysis. Tumor size was evaluated using various MRI techniques, including T1-weighted, T2-weighted, diffusion-weighted imaging (DWI) and gadolinium-enhanced imaging. MRI assessments were conducted by a single radiologist and interpreted according to the Response Assessment in Neuro-Oncology (RANO) criteria. The follow upping MRI and clinical assessment involved several key time points: recruitment, after the first cycle of injections, and at 1, 3, 6, and 12 months subsequent the last injection. Additional scans were conducted as needed based on clinical indications. The clinical status of patients was evaluated through comprehensive history-taking and physical examinations conducted by the oncologist. In the present study the complete elimination of the tumor was considered a complete response (CR) without any new lesion and stability in terms of clinical status. A $\geq$ 50% decrease in the greatest perpendicular diameter of enhancing lesions compared with baseline, without any new lesions and no increase in no enhancing tumors, was considered a partial response (PR), progressive disease (PD) was defined as a $\geq$ 25% increase in the sum of the longest diameters of target lesions or the appearance of new lesions, and stable disease (SD) was defined as neither sufficient shrinkage to qualify for PR nor a sufficient increase to qualify for PD. To evaluate the changes in the CSF, 2 mL samples were collected prior to each injection. The collected samples were subjected to various tests, including hematology tests to determine red blood cell (RBC) counts, white blood cell (WBC) counts, differential counts (diff), and CSF smears. Additionally, biochemical tests were conducted to measure the concentrations of glucose, LDH, and total protein in the CSF. The bacteriology tests involved Gram staining and bacterial culture to identify the presence of any bacteria. Furthermore, immunophenotyping by using flow cytometry focused on T cells, NK cells, and NKT cells, and cytokine levels (INF-Y and TNF-α) were measured using ELISA. Patient survival outcomes, including overall survival (OS) and progression-free survival (PFS), were monitored at the end of an 18-month follow-up period.

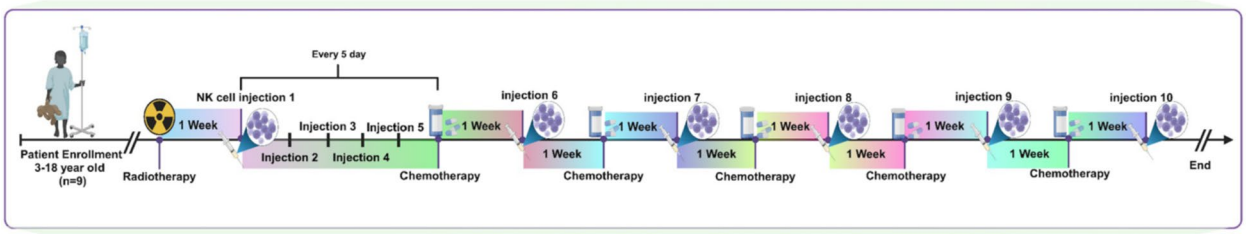

Fig. 1 Schematic presentation of treatment protocol at intervention group. Patients who undergoing chemotherapy received weekly NK up to five injection, scheduled with their chemotherapy sessions. The initial dose was given one week after the final chemotherapy session to minimize chemotherapy's impact on NK cell survival. For patients who were undergoing radiotherapy, the first NK cell cycle also was began one week post-radiotherapy, with weekly injections thereafter up to five injections. An MRI scan was performed one month after the fifth injection to check for any new neurological lesions or disease progression. If new lesions or disease progression were detected, NK cell injections were terminated. If no progression was observed, a second cycle of NK cell injections was administered between chemotherapy courses, spaced a week apart

NK cell purification and characterization

Peripheral blood mononuclear cells (PBMCs) were collected from healthy volunteer donors via leukapheresis after providing written consent. The health status of the donors was checked by history and laboratory tests, including CBC diff, BUN, Cr, ALT, AST, bilirubin (T, D), HbA1 C, VDRL, HIV Ab, HBS Ag, HBS Ab, HCV Ab, CMV Ab (IgG, IgM), EBV Ab (IgG, IgM), and HTLV-I Ab. Owing to the COVID-19 pandemic during the study, eligibility criteria for donors also included the administration of two doses of the SARS-CoV-2 vaccine and a negative QRT–PCR test 48 h prior to leukapheresis. Using the OPTIA® Spectra apheresis system (Terumo BCT, EUROPE N.V., Belgium), up to 280 ml (total nucleotide cell count $23 \times 10^9 \pm 10.3$) of peripheral blood was collected and further processed via the Miltenyi Biotec CliniMACS Plus cell selection system via the CD56 + enrichment program, following the manufacturer's recommendations (Miltenyi Biotech, Bergisch Gladbach, Germany). The isolated cells were aliquoted and cryopreserved at −195 °C before injection. Multiple tests were conducted to control the quality of the selected NK cells, including sterility via the BacT/ALERT microbial detection system; cell counting and viability via a Neucleo Counter SCC-100™ (Denmark); and immunophenotyping to evaluate the percentages of CD56-, CD3-, CD19-, CD14-, CD56-, CD16-, NKG2D-, and NKG2 A-positive cells via BD FACS Calibur™ (Germany).

The release specification criteria were as follows: total nucleated cell viability >90%, negative microbiological test results, percentage of CD56 +/16 + cells ≥90%, CD3 + cells ≤5%, CD14 + cells ≤5%, and CD19 + cells ≤5%. Table 1 summarizes the characterization protocol for purified NK cells. In the present study, the percentages of NKp30 and NKG2D as activator markers were >10%, ≥24%, and ≥86%, respectively. Although NKG2 A, an inhibitory marker, was expressed in less than 30% of the total NK cells, standardized quality controls were applied at all steps of the injections. A total of 40–50 million cells were thawed and resuspended in 1 mL of normal saline containing 10% HSA at room temperature. NK cells were activated with 20 ng/mL IL-15 for 1 h and then transported to the hospital. The recovery rate of the cryopreserved cells was greater than 90% (Supplementary Table 1).

Table 1 Characterization of allogenic purified NK cells

Quality test	Mean + SD
Purity of total isolated $CD56^+$ $CD16^+$ cells (%)	95.5 ± 1.82
Contamination of $CD56^-$ $CD3^+$ T cells (%)	2.52 ± 2.27
NKG2D (%)	91.02 ± 4.61
NKp30 (%)	29.14 ± 18.3
NKp46 (%)	32.96 ± 8.1
NKG2 A (%)	16.69 ± 9.65
CD14 (%)	0.75 ± 1.08
CD19 (%)	0 ± 0.015

Statistical analysis

Continuous variables are presented as the mean accompanied by the standard deviation (SD), whereas qualitative variables are expressed as frequency percentages. Adverse events are reported in descriptive statistics as incidence and frequency percentages. Correlations between the variables were analyzed via the Mann–Whitney test. Two-way ANOVA was used to analyze the radiological data. Clinical evaluations were performed in a qualitative manner. Survival analyses were performed via the Kaplan–Meier method, and the results are presented in the related charts. To evaluate different parameters in CSF, Wilcoxon, Mann–Whitney, and Friedman tests were performed via SPSS20 software (IBM, NY, USA), and $p < 0.05$ was considered significant.

Results

Patient demographic data

Seventeen patients underwent screening for eligibility, of which nine met the inclusion criteria (Fig. 2). The baseline characteristics, recurrence episodes, and treatment cycles for these patients are summarized in Table 2.

In order to offer a comparative perspective, six retrospective patients who had undergone conventional therapy at the same center and matched for disease status were selected (Fig. 2). In total, 15 patients (9 in the intervention group and 6 in the retrospective control group) were enrolled: 7 males (47%) and 8 females (53%), aged 3 to 18 years (mean ± SD: 9.05 ± 4.1 years). The Lansky index was assessed for each patient according to the standard protocol [13]. The intervention group comprised patients with relapsed-refractory glioma and diffuse intrinsic pontine glioma (DIPG) ($n = 7$, Ependymoma ($n = 1$), and Pineoblastoma ($n = 1$).

The retrospective control group consisted of patients with high-grade gliomas ($n = 5$) and ependymoma ($n = 1$). For those patients with unresectable glioma ($n = 4$), obtaining pathology samples through surgery or biopsy was not applicable and selected on the basis of the neurosurgeon's assessment and imaging finding. Patients in both groups received varying cycles of chemotherapy and displayed refractory response. However, detailed treatment records for the retrospective control group were unavailable. Seven patients in the intervention group received ten injections of active NK cells, whereas two patients received nine injections due to disease

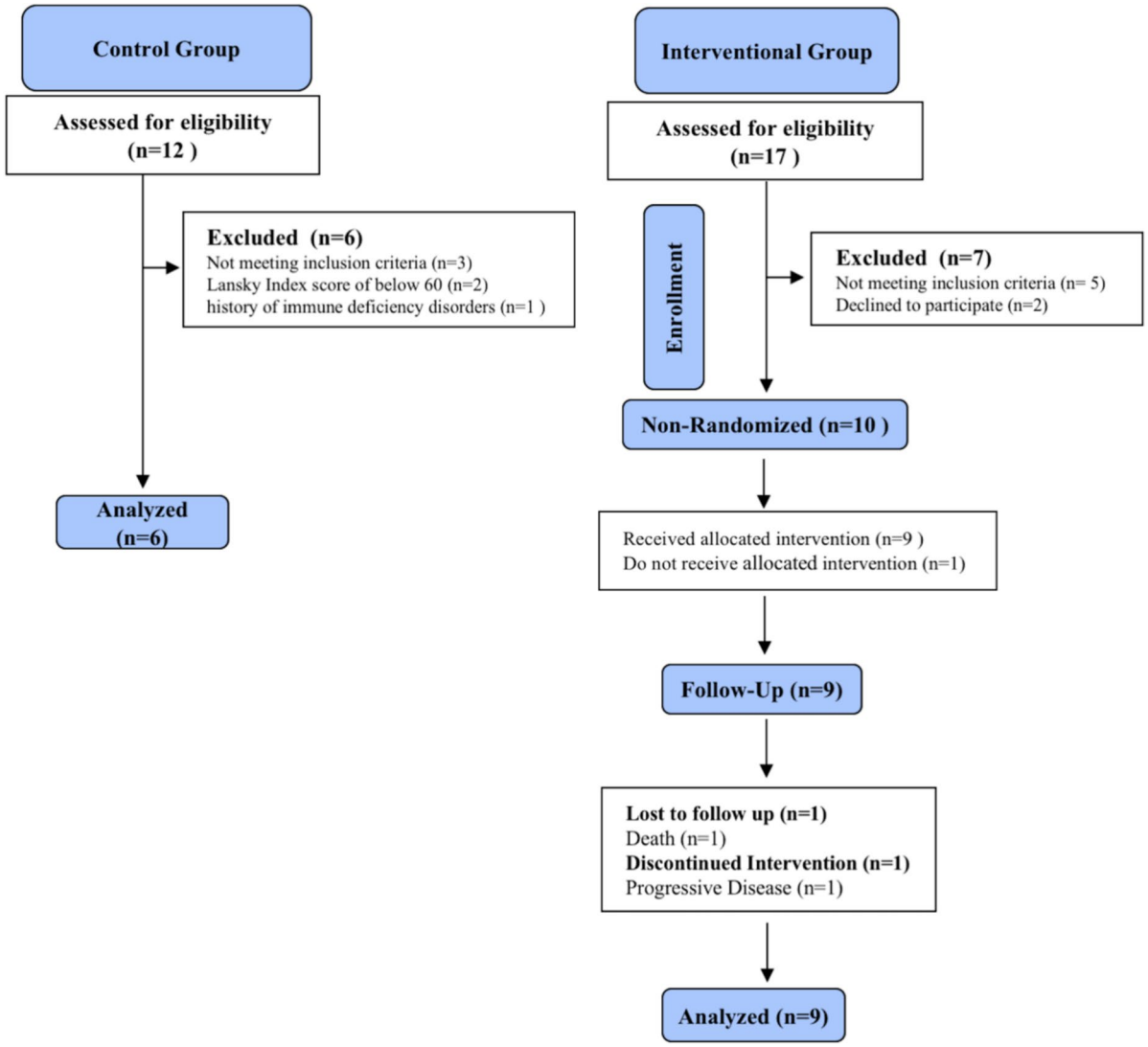

Fig. 2 CONSORT diagram of patients. Retrospective controls were selected and added at the end of the study from patients who had the same disease status and chemo-/radiotherapy to the interventional group

progression and/or lumbar puncture contraindication (Table 2). For Case 5 in the intervention group, the initial status was classified as Progressive Disease (PD). Following disease progression, immunotherapy with nivolumab and Avastin (bevacizumab) was introduced, resulting in tumor size reduction and suggesting a promising response after treatment adjustments.

Adverse effects

In this trial, we demonstrated the safety of administering 88 intrathecal injections of allogenic NK cells across nine intervention group, with no apparent study-related serious adverse events (SAEs) (Fig. 3A, B). All adverse events (AEs) were mild (grades 1 and 2), predominantly associated with lumbar puncture procedures, and occurred within 48 h post-injection. However, Patient 9 experienced a seizure two hours after the 10 th injection, coinciding with the first administration of a new brand of potassium citrate used for portal vein exchange. This event could potentially be considered a treatment-related SAE, as potassium citrate is known to induce hypocalcemia, which may trigger seizures. Nevertheless, after thorough evaluation, the DSMB committee at the Royan institution concluded that the seizure was not causally related to NK cell injections. The most prevalent AEs were headache [29% (17% grade 1 and 13% grade 2)], fever and chills [21% (17% grade 1 and 4% grade 2)], vomiting [13% grade 2], and back pain [12% (4% grade 1

Table 2 Baseline characteristics of the patients enrolled in the (A) intervention group and (B) retrospective control group

A	Case	Gender	Age (year)	Lansky Index	Pathology	Condition	Tumor location	Recurrence episodes	Treatment cycles (N)	Injections (NO)
Intervention	1	Female	6	90	Ependymoma, grade 3	Relapsed-Refractory	Brain stem and cerebellum	2	15	10
	2	Male	13	70	High grade glioma	Relapsed-Refractory glioma	Frontoparietal lobe	2	4	9
	3	Male	11	70	No biopsy	DIPG	Pons	0	14	9
	4	Female	5	90	No biopsy	DIPG	Pons	2	8	10
	5	Female	4	100	No biopsy	Unresectable Glioma	Suprasellar region and basal ganglia	3	6	10
	6	Female	10	70	No biopsy	Unresectable Glioma	Diffuse glioma with infiltration to optic chiasma	4	6	10
	7	Male	9	80	High grade glioma	Relapsed-Refractory glioma	Midbrain	3	12	10
	8	Male	3	90	High grade glioma	Relapsed-Refractory glioma	Pons	2	8	10
	9	Male	6	60	Pineoblastoma, grade 4	Unresectable tumor	Mid Brain	9	4	10
B	**Case**	**Gender**	**Age (year)**	**Lansky index**	**Pathology**	**Condition**	**Tumor location**			
Historical Control	1	Male	14	70	Ependymoma	Recurrent-Refractory	Posterior Fossa			
	2	Male	14	60	High grade glioma	DIPG	Diffuse glioma with progression to parietal lobe			
	3	Female	6	80	High grade glioma	DIPG	Brian Stem			
	4	Male	14	60	High grade glioma	DIPG	Pons			
	5	Female	9	60	High grade glioma	Recurrent-Refractory glioma	Pons			
	6	Male	6	60	High grade glioma	Unresectable Glioma	Pons			

N/A Not Applicable

A

Case	Tumor location	Adverse events	How long after injection?	Treatment-related	Injection	Grade
1	Brain stem & cerebellum	Hypothyroidism Death	140 h 6 M	NO NO	6	1
2	Frontoparietal lobe	Headache Mucositis, oral Muscle weakness Death*	48 h 96 h 72 h 12 M	Possible NO NO NO	1 4 9	1 2 2
3	Pons	Headache Vomiting Dysphasia Depressed consciousness* Seizure* Seizure* Ventricular tachycardia* Asystole*	24 h 24 h 72 h 312 h 312 h 165 h 90 h 167	Possible Possible NO NO NO NO NO NO NO	2 2 4 5 5 8 9 9	2 2 2 4 3 4 4 5
4	Pons	Rash Cough	72 h 120 h	Possible NO	1 8	1 1
5	Suprasellar region	Irritability Vomiting Vomiting	48 h 8 h 10 h	NO Yes Yes	5 8 9	1 2 2
6	Optic chiasma	Back pain Gait disturbance Abdominal pain Fever & Chills Upper respiratory infection Headache Fever Back pain Headache Fever Back pain Headache	12 h 36 h 12 h 166 h 100 h 8 h 8 h 4 h 10 h 10 h 12 h 16 h	Yes Yes NO NO NO Yes Yes Yes Yes Yes Yes Yes	3 3 3 4 5 6 6 7 7 7 8 8	2 2 1 1 1 2 1 2 2 2 1 1
7	Mid brain	Headache Fever & Chills Headache Malaise Fever	12 h 14 h 4 h 14 h 10 h	Yes Yes Yes Yes Yes	2 5 6 6 6	1 1 1 1 1
8	Pons	Upper respiratory infection	24 h	NO	8	1
9	3rd and 4th ventricle	Diarrhea COVID infection Seizure* Death	80 h 72 h 2 h 5 M	NO NO Possible NO	1 4 10	1 1 2

B

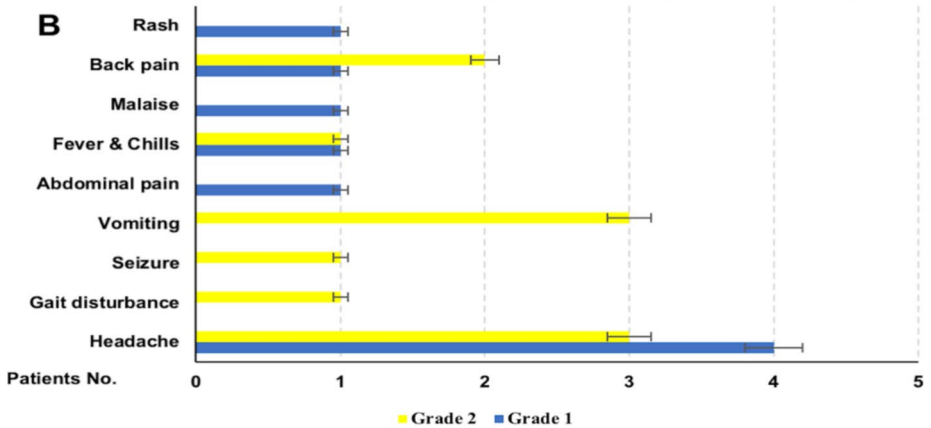

Fig. 3 **A** Description of adverse events after each infusion, grading and relation to treatment. **B** The bar plot indicates the number of patients with the most common symptoms

and 8% grade 2)]. Although headache was the most frequent symptom, all cases resolved within three days post-injection. No correlation was identified between tumor location and AEs occurrence. Furthermore, four fatalities were reported in the intervention group, —two due to tumor progression and two attributed to COVID-19 infection. Following a detailed review, the Royan DSMB committee concluded that these fatalities were not associated with the intervention.

Patient follow-up and survival

Among the nine patients who were administered active allogenic NK cells, five survived for 18 months after the last injection and until the results were reported (Aug. 07. 2024). Using clinical and radiological criteria (Fig. 4A), the study demonstrated a partial response (PR) in 1 and progressive disease in 1 out of the five survived patients during the follow-up period. Stable disease (SD) was identified in three patients, while 2 patients died as a result of progressive disease (PD). Additionally, we lost 2 patients due to infectious diseases (patient number 1 due to COVID-19 infection and patient number 9 due to fever and neutropenia and fungal infection of the shunt while in a partial response phase) (Fig. 4A, B). some deaths in the intervention group may be related to COVID-19 or other infections due to the specific healthcare conditions during that period. Majority of patients experienced 2 to 4 episodes of recurrence before receiving NK cells, except patient 3 who did not report any recurrence but was refractory (Tables 2, 3 and 4). On the other hand, the presence of LMS was not detected in this group until the data were reported on Aug.07. 2024 (28 months after the last injection) (Tables 3 and 4). The Kaplan–Meier survival analysis revealed that the overall survival (OS) and progression-free survival (PFS) rates were both 45% during the 12-month follow-up period following the last injection of NK cells (Fig. 4B). Conversely, most patients in the retrospective group experienced tumor regrowth and local metastatic spread (LMS) during the follow-up period, including two patients with spinal cord seeding, one patient with cerebrospinal fluid (CSF) seeding, one patient who developed LMS, and two patients with localized relapses. Detailed information regarding the number of recurrences and treatment cycles was not available for this group in the study.

Our data revealed that the median survival of patients in the interventional group was significantly greater than that of patients in the retrospective control group since tumor diagnosis (48 months vs. 18.5) (Fig. 4C).

CSF analysis of the intervention group

This study evaluated the immune cell phenotype and the levels of IFN-γ and TNF-α in the CSF before and after the last injection. Our results revealed a significant reduction in IFN-γ ($P = 0.003$) and TNF-α ($P = 0.01$) levels after the last NK cell injection (Table 5), especially in patients who were alive and good responders to NK cell therapy. Moreover, we found significant changes in the cellular fractions of CSF after NK cell injection. Interestingly, the percentages of NK and T cells strongly increased after ten injections (Table 5, $P < 0.003$ and < 0.006). However, the level of NKT cells did not change after NK cell injection ($P > 0.7$). Furthermore, the number of immune cells in the CSF at the beginning of the study was poor and continuously increased following NK cell administration (Table 5).

Discussion

The most critical challenges in gliomas are tumor resection limitations, side effects, resistance to TMZ/radiotherapy regimens, and recurrence, highlighting the need to improve novel therapeutic approaches. Several studies have demonstrated the potential of NK cells as effectors of brain tumors [14–17]. Moreover, we previously showed that NK cells efficiently eliminate glioblastoma in a rat model [10] and that post-surgery administration of activated haploidentical NK cells directly into the tumor site is safe and tolerable and could increase the overall survival of refractory/recurrent gliomas. During 10 months of follow-up, although tumor regrowth was not observed at the administration site, some patients experienced tumor recurrence in other parts of the CNS [11]. In the present study, we improved the procedures and introduced a novel administration protocol for NK cell therapy as an alternative therapy, in which 10 weekly injections of a defined number of NK cells (40–50 million

(See figure on next page.)

Fig. 4 **A** MRI images of the tumors revealed tumor progression in patients 1 and 2, a stable tumor size in patient 7, and a partial response in patients 5 and 9. MRI sequences were T1-weighted (P1, P2, P5, P9) and T2-weighted (P7) **B** (Right) A bar plot summarizing the disease status of all 9 enrolled patients in the intervention group. *; Patients 1 and 9 died from infections unrelated to NK cell injections. (Middle) The overall survival plot for the interventional group was calculated from the last injection of NK cells to the 12-month follow-up. (Left) Progression-free survival plot for patients receiving 10 cycles of NK cells weekly during radio/chemotherapy. **C** A median survival comparison was performed between the intervention and retrospective control groups via the Mann–Whitney U test, which revealed a significant difference in patient survival between the two groups

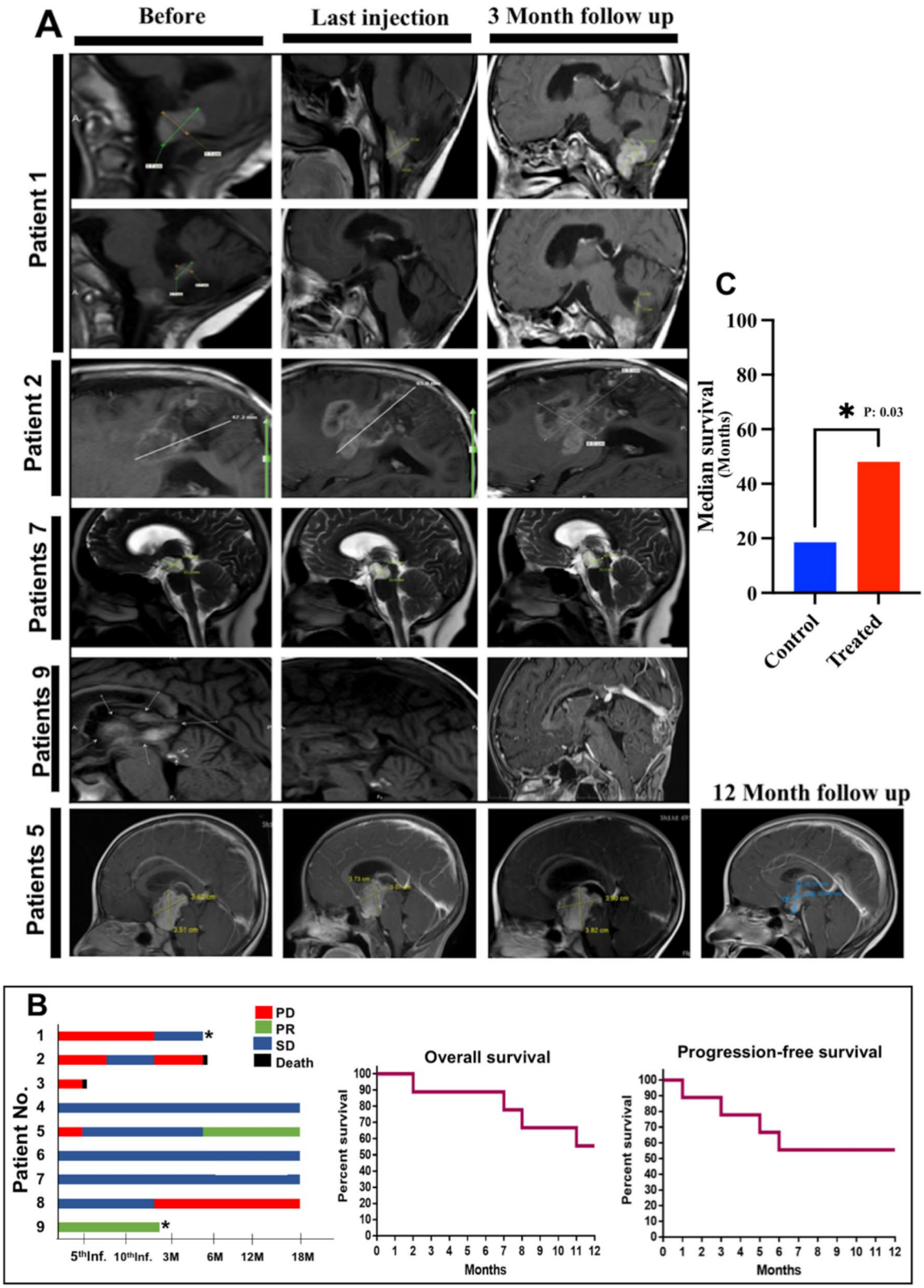

Fig. 4 (See legend on previous page.)

Table 3 Treatment response, disease status, recurrence, and overall survival of patients receiving 9–10 weekly intrathecal injections of active Natural Killer cells

Patient No	Tumor size (CM)							
	Before	Post 5 Inf	Post 10 Inf	Follow up			Recurrence/ CSF Spreading	OS* /Status (Month)
				3 M	6 M	12 M		
1	Cerebellar T 22 × 16 × 10.5	Cerebellar T 25 × 16 × 12.5	Cerebellar T 30 × 25 × 20	Cerebellar T 40 × 30 × 25	N/A	N/A	NO	65/SD
	3 th Ventricle T 12.5 × 7 × 6	3 th Ventricle T 12.5 × 7 × 6	3 th Ventricle T 5 × 1 × 1	3 th Ventricle T 5 × 1 × 1	Because of Death			
2	49 × 23 × 20 fronto-parietal lobe	55 × 30 × 25	65 × 40 × 35	65 × 40 × 35	105 × 95 × 85	105 × 95 × 85	NO	21/PD
3	60 × 40 pons	60 × 40 New Lesions 19 × 15 22 × 17	N/A Because of Death	N/A	N/A	N/A	1 New lesion	17/PD
4	37 × 37 × 35 pons	40 × 40 × 35	40 × 40 × 35	-	40 × 40 × 35	40 × 40 × 35	NO	39/SD
5	37 × 37 × 35 pons	40 × 30 × 25	40 × 30 × 25	40 × 30 × 25	40 × 30 × 25	25 × 13 × 10	NO	71/PR
6	25 × 20 × 15 Diffuse glioma with infiltration to optic chiasma	25 × 20 × 15	25 × 20 × 15	25 × 20 × 15	-	25 × 20 × 15	NO	111/SD
7	25 × 22 × 20 Midbrain	25 × 22 × 20	25 × 22 × 20	-	25 × 22 × 20	25 × 22 × 20	NO	70/SD
8	No measurable, pons	No measurable	No measurable	13 × 11 × 11	11 × 10 × 10	17 × 15 × 12		48/PD
9	Midbrain 3thventricle T. 35 × 25 × 20	3 th Ventricle T. 0 × 0 × 0	3 th Ventricle T. 0 × 0 × 0	3 th Ventricle T. 0 × 0 × 0	N/A	N/A	NO	20/CR
	Seeding cervical spinal cord Subarachnoid	Incompetence of Seeding around cervical spinal cord Subarachnoid	Stability of Seeding cells	Stability of Seeding cells	Because of Death due to fever and neutropenia			

* means multiplication of numbers

Table 4 Treatment response, disease status, recurrence, and Overall survival of patients in the retrospective control group

Patient No	Tumor size (CM)			Recurrence episodes/CSF spreading	Status	Overall survival* (Month)
	Before	Follow up 2	Follow up 1			
1	53*38	14*9.5	17*11	NDD/Yes	Alive SD	27
2	15×14.5	58×43	43×40×33	1 new lesion/No	Alive PD	28
3	35×25×50	37×44	40×35	1 New lesion Leptomeninges/No	Death PD	8
4	45×40×64	37×45×90	N/A	NDD/yes	Death PD	34
5	50×47×39	58×50	N/A	NDD/yes	Death PD	5
6	50×58	N/A	N/A	NDD/Yes	Death PD	10

* means multiplication of numbers

Table 5 Cerebrospinal fluid (CSF) analysis to evaluate the cellular and immune factors after last injection. The level of IFN-γ and TNF-α and the percentage of NK, NKT and T cells in CSF of patients before the first and after the last injection of NK cells (*; death due to infection unrelated to the intervention)

Case No (Status)		1 (SD*)	2 (Death)	3 (Death)	4 (SD)	5 (PR)	6 (SD)	7 (SD)	8 (PD)	9 (PR*)	Mean + SD	Sig
Factors												
IFN-γ (pg/ml)	**Before**	70.60	61.95	79.25	81.35	101.25	74.70	74.73	94.30	113.20	83.48 ± 16.28	0.003
	After	19.50	41.35	33.75	14.20	25.53	29.30	27.75	38.95	23.30	26.18 ± 8.82	
TNF-α (pg/ml)	**Before**	14.63	33.04	39.08	44.92	53.96	30.63	49.44	49.00	63.17	41.99 ± 14.45	0.01
	After	13.41	35.70	29.20	14.83	49.58	15.45	26.41	37.58	17.20	26.6 ± 12.56	
%NK Cell	**Before**	0.00	0.00	0.00	0.00	2.60	1.13	1.10	0.00	0.80	0.63 ± 0.89	0.003
	After	3.90	0.03	0.02	1.20	5.40	4.10	3.80	2.40	0.90	2.42 ± 1.97	
%NKT Cell	**Before**	0.00	0.00	6.90	0.00	0.80	0.00	0.30	0.00	2.40	1.16 ± 2.29	0.71
	After	1.30	0.00	0.00	6.00	0.00	3.80	8.20	1.00	0.70	2.33 ± 2.99	
%T Cell	**Before**	1	1	1	4.10	21.40	1.00	7.20	1	26.40	7.12 ± 9.82	0.006
	After	87.30	88.70	74.00	62.20	45.60	63.10	76.10	86.00	28.30	67.92 ± 20.5	

cells) in combination with chemo-/radiotherapy were scheduled. Moreover, we changed the route of administration of NK cells from intralesional injection to intrathecal injection to increase the ability of circulation in the central nervous system (CNS) and reduce the risk of LMS. On the basis of the off-the-shelf strategy for the use of NK cells, we applied allogenic NK cells that were cryopreserved and banked under standard conditions. Therefore, we conducted the present study to evaluate the safety and feasibility of multiple injections of activated allogenic NK cells in pediatric patients with recurrent or chemo-/radiotherapy-resistant gliomas. To better understand the antitumor effect of NK cells as a secondary outcome, a retrospective control group that received standard treatment was added to the present study.

Our results demonstrated that weekly intrathecal injection of allogenic NK cells up to 10 times during chemotherapy and after radiotherapy is safe. Mild adverse events, including fever, chills, headache, back pain, and vomiting, were reported and resolved three days after the injection. However, a seizure occurred 2 h after the last injection in one patient, which could be considered a possible treatment-related serious adverse event (SAE). Although there are no reports evaluating the safety of intrathecal injections of NK cells in patients with brain tumors, Khazal et al. noted that immunotherapy using NK cells for solid tumors is generally considered safe and well tolerated, with mostly transient low-grade side effects such as fever, weight loss and neurotoxicity [18, 19].

Another notable finding in the present study was the reduction in recurrence episodes following NK cell injections in patients who responded to the treatment. Interestingly, 5 out of 9 patients exhibited a response to this therapy (3 with SD and 2 with PR). Among the patients who experienced loss during the study, two died due to infection, while their disease remained stable or showed a partial response. Overall survival improved to 28 months postintervention (48 months after diagnosis), without any reported cases of LMS in this group. In contrast, the retrospective control group exhibited a higher incidence of LMS, with an overall survival of approximately 18.5 months after diagnosis.

CSF analysis added novel data in the present study that can direct us to an approach that predicts the response to NK-cell therapy. The most important factors underlying these changes were immune cells. Most patients do not have any WBCs in the CSF before the injection of NK cells. However, the WBC count increased after NK cell injection. Among immune cells, NK and T cells showed dramatic changes in the CSF after treatment, especially in patients who achieved SD, PR, or CR. These results are in accordance with the results reported previously by Khatua S. et al. [18]. Although few studies have determined the cellular phenotype after NK cell injection, Greer and colleagues reported that the presence of tumor cells in the CSF was correlated with a decrease in the lymphocyte number and an increase in the monocyte count. Patients who do not survive because of tumor progression have a reduced proportion of lymphocytes in their CSF [20].

Furthermore, more activated NK cells are found in low-grade gliomas than in high-grade gliomas [21], and dysfunction of T cells has been reported in glioblastoma patients [22, 23].

On the other hand, the reduction in IFN-γ and TNF-α levels in the CSF of patients after NK cell injection, especially in those patients who responded better to

therapy and are alive, suggests a role for these cytokines in tumor progression. In 2020, Weiun reported that NK cells that infiltrated into tumor lesions presented elevated levels of CXC chemokine receptor 3 (CXCR3) and reduced levels of IFN-γ. This study investigated the immunosuppressive microenvironment in glioblastoma patients at the single-cell level [24].

The present study had several limitations, including the small number of patients and heterogeneity among the participants. Difficulties in data collection from the retrospective control group, as well as limited access to CSF analysis, hindered effective comparison of the results. These limitations could be addressed in future studies by selecting an appropriate control group and extending the follow-up period.

Conclusion

Administering allogenic NK cells through multiple intrathecal injections is considered safe and feasible for pediatric patients with refractory/recurrent high-grade gliomas. The addition of NK cells as an alternative therapy following conventional therapies has improved the overall survival rate of patients. Additionally, this approach has been associated with the inhibition of tumor growth and a reduction in the occurrence of LMS. However, the efficacy of NK cells in targeting glioma needs to be confirmed in a phase II clinical trial. Furthermore, the observed enhancement of NK and T cells in the CSF following NK cell injection, alongside the reduction in IFN-γ and TNF-α levels, represents a novel finding. Nevertheless, the limited sample size prohibits its conclusive conclusions regarding its clinical significance. Our initial observations indicate that patients who showed an increase in NK and T cells and a decrease in IFN-γ and TNF-α in the CSF experienced improved survival outcomes. Although these results are promising, further investigation with larger sample size is necessary to establish their robust clinical implications.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12885-025-14314-6.

Supplementary Material 1.

Acknowledgements

The authors would like to express their gratitude to Dr. M. Zarrabi, the CEO of Royan Stem Cell Co., and his team, M. Mohammad, M.H. Ahmadi, and all the fellow colleagues of Royan Stem Cell Technology Co., for their collaboration and support in the use of the clean room facility. The authors would also like to thank Dr. J. Firouzi and M. Azimi for GLP training. We also acknowledge Prof. A.H. Shahverdi, the head of the Royan Institute; Prof. H. Baharvand, the director of the Royan Institute for Stem Cell Biology and Technology; Prof. A.A., the Hamidieh director of Pediatric Cell and Gene Therapy Research Center; and the Gene, Cell & Tissue Research Institute, Tehran University of Medical Sciences, who supported us in this study. We would like to express our sincere gratitude to all the nurses and personnel of Rasoul Akram Hospital for supporting the patients and thank the patients and their families who agreed to participate in this study. This study was funded by Kian Immune Cell Co., Royan Lutose Grant Body, National Council for Development of Regenerative Medicine and Stem Cell Technologies.

Authors' contributions

HM; Investigation, Project Administration and Writing – original draft, AI; Data curation, Investigation and Writing- review & editing, YN; Visualization and Writing – review & editing, PSh; Software, Visualization and Writing – review & editing, DD; Investigation and Writing – review & editing, AAA; Validation, Visualization and Writing – review & editing, AT; Methodology, Validation and Writing – review & editing, HH; Software, Validation, Visualization and Writing – review & editing, MV; Validation and Writing – review & editing, PF; Methodology and Writing – original draft, MA; Methodology and Software, SU; Formal Analysis, AB; Software and Writing – original draft, AKh; Data Curation, Formal Analysis and Software, MF; Conceptualization, Methodology, Supervision and Writing – review & editing, ME; Conceptualization, Methodology, Supervision and Writing – review & editing. All the authors have read and approved the final article.

Data availability

The data that support the findings of this study are available from the corresponding author upon request.

Declarations

Ethics approval and consent to participate

This study was conducted in accordance with the principles of the Declaration of Helsinki and was approved by the Royan institutional scientific review board and the Royan ethical committee in compliance with national regulations (IR.ACECR.ROYAN.REC.1400.077). All patients signed written informed consent to participate in research and for publication prior to their inclusion in the present study.

Consent to publication

Written informed consent was obtained from all participants for publication purpose.

Competing interests

The authors declare no competing interests.

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Received: 4 February 2025 Accepted: 12 May 2025
Published online: 27 May 2025

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