The Safety and Feasibility of Intra Surgical Cavity or intra Cerebrospinal Fluid (CSF) Injection of Haploidentical Activated NK Cells in Patients with Recurrent Glioblastoma Multiforme and Brain Tumors

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Case Report

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Abstract

Despite multi-modal therapies for patients with malignant brain tumors, their median survival is < 2 year. Recently, NK cells provided to cancer immune surveillance through their direct natural cytotoxicity, by modulating dendritic cells to enhance the presentation of tumor antigens and regulating T-cell mediated antitumor responses. However, the success of this treatment modality in brain tumors is unclear. The main reasons are the brain tumor microenvironment, approach of NK cell preparation and administration and the donor selection. Our, previous study showed that intracranial injection of activated haploidentical NK cells resulted in the eradication of glioblastoma tumor masses in the animal model. The animals had no evidence of tumor recurrence after treatment, and all tumor-related complications resolved after treatment. Therefore, in the present study, we evaluated the safety of intra surgical cavity or intra cerebrospinal fluid (CSF) Injection of ex vivo activated haploidentical NK cells in six patients with recurrent glioblastoma multiform (GBM) and malignant brain tumors resistance to chemo/radio therapy. The results showed that local administration of the haploidentical NK cells in malignant brain tumors is safe, feasible, tolerated at higher dose and also is cost effective.

Introduction

The average cancer incidence of brain and nervous system worldwide in 2020 was about 308000 cases (in Iran it was about 6180 at the same time) that will rich to 335000 patients (in Iran it would be around 7160 cases) in 2025[1]. Among brain tumors, glioblastoma multiforme (GBM) is the most aggressive, WHO grade IV glioma de novo brain tumors with a median survival of three months if untreated and constitute for almost 60% and 3–15% of all malignant primary brain tumors in adults and children, respectively [2–4]. The median survival rate is poor with a 14.6-month survival [5, 6]. Current combined standard of treatment for newly diagnosed brain tumors including GBM consist of maximal safe surgical resection of tumor, where possible, followed by concurrent radiotherapy with temozolomide (TMZ), and then adjuvant chemotherapy with temozolomide (TMZ). Yet, these conventional strategies provide only temporary benefits, and the tumor eventually becomes resistant, progresses and relapses almost all the time[7, 8].

Tumor heterogeneity and presence of blood brain barrier (BBB) intricate specific tumor microenvironment (TME) in these patients including diffuse spread of tumor cells, abnormal blood vessels of tumor, specific immune escape and also some of obstacles contribute to ineffective therapeutics delivery and multi drug resistance[9, 10]. Therefore, there is an urgent unmet need to investigate novel interventions that turn GBM unique tumor microenvironment features into our advantage to have optimal therapies with long lasting remission of this devastating malignancy and increase survival rate.

Immune-cell based therapies applying various immune cells such as T-cells, dendritic cells (DCs) and natural killer cells (NK cells) are advancing quickly as potential adjunctive interventions in combination with conventional approaches or targeted therapies to potentiate the efficacy of treatment against malignant brain tumors including GBM[12, 13]. Among immune cells, administration of NK cells has been indicated its potential in developing new promising anti-brain tumor treatments, in vitro and in vivo[14, 15]. They can distinguish stressed target cells including malignant cells independent of antibody and major histocompatibility complex (MHC) and without previous exposure to antigenic peptides with prompt consequent cytotoxic reaction [16]. This function particularly is important when considering brain tumor immunosuppressive microenvironment [17, 18]. Intriguingly, it has been shown that NK cells can recognize and destroy human glioblastoma stem cells (GSC), one of the main players of drug resistance and tumor relapse[19, 20].

The specific GBM microenvironment and the presence of blood brain barrier (BBB), limit the success of immune cell therapies. Different routes of administration including systemic, intracavitary, intracranial, intralesional, intrathecal were applied to increase the efficient NK cells homing to tumor site, prolonged consistency, cell- cell contact with tumor cells and chemotaxis[21]. Our previous study in animal models showed that injection of activated NK cells into tumor cavity, not only eradicate tumors but also increase overall survival of rat bearing GBM compared to systemic administration[14]. Therefore, the current phase I clinical study aims to evaluate the safety and feasibility of intra surgical bed or intra CSF administration of haploidentical activated NK cells in patients with rGBM and malignant brain tumors resistance to treatment.

Materials And Methods

Study design

This was a single-arm, open-label, investigator-initiated phase I clinical trial in recurrent malignant brain tumors including GBM. The study included six patients. Mean total follow up was 8.08 months (2 weeks-18 months). The study was approved by Royan ethical committee (IR.ACECR.ROYAN.REC.1398.261), registered at Iranian Registry of Clinical Trials (IRCT20170122032121N5) and also in international registry of clinical trials (NCT05108012). The written informed consent was obtained from patients or their legal guardians and healthy donors before participating in this study.

Patient and haploidentical Donor Selection

Patient and donor recruitment and research progress were conducted by a member of the clinical team based on inclusion criteria at Children's Medical Center hospital (CMC), Tehran, Iran, and Golestan medical hospital, Iran. (Table 1, supplementary).

Isolation and Activation of CD56+/ CD16 + cells

The CD56/16 positive cells were isolated using CD56 Reagent (Miltenyi Biotec GmbH, Germany) and CliniMACS instrument according to manufacturer’s instruction (Protocol: Enrichment of CD56 positive cells). The cell cryopreservation was done using Planner instrument (Kryo 560 – 16, USA) and finally, cryovials transfer into ~ 198°C until use.

The isolated NK cells were activated using overnight incubation with 10 ng/ml of IL 15. The fresh isolated cells were used as the first injection for each patient, and the next injections were done using the cryopreserved cells.
Quality control

The quality of CD56+/16+ isolated cells was assessed in several steps using various methods. (Table 2, supplementary).

Evaluation of Cytotoxic Activity

To assess cytotoxicity activity of isolated NK cells; cancer cells (U251 (malignant GBM), SKOV3 (ovarian adenocarcinoma line) and K562 (chronic myelogenous leukemia)) line were stained using calcine-AM and then were cultured in 24 wells plate with cancer cells at ratio of 1/3 (Effector/target cells) at 37°C. Next, the analysis was done using flow cytometry. Propidium iodide (PI) staining was done for all samples.

Interferon gamma (IFN-γ) release assay

Interferon Gamma (IFN-γ) release assay was performed to indicate secretory power of activated NK cell against cancer cells. The cell free supernatant of activated (with IL-15), inactive NK cell, and cells that co-cultured with tumor cells were collected after overnight incubation at 37°C under 5% CO2 in air. All samples were assessed by ELISA method to determine the IFN-γ concentration.

NK Cell infusion to the patients

Patients who participated in the study received NK Cells three times, on a weekly interval. The first injection was done two weeks after surgery using freshly activated cells and the others were cryopreserved cells which were activated post thawing. Depending on tumor size, 2x10⁶ up to100x10⁶ activated NK cells were injected directly into the tumor bedside using implanted reservoirs. The reservoirs were implanted at the time of surgery, after tumor resection, in three cases. In other three patients, the activated NK cells were delivered into CSF circulation through an intraventricular catheter and intrathecal route, respectively. Graphical abstract shows a schematic picture of clinical trial procedures.

Patients follow-up and adverse event assessment

All follow-up process was monitored and recorded by the neurosurgeon who performed the injection and an independent clinical assessor. (Table 3, supplementary).

Statistical analysis

Safety data were summarized using descriptive statistics. All data were expressed as the mean ± SD and were evaluated by paired T-Test or Mann–Whitney test, as appropriate. A probability value of p < 0.05 was considered statistically significant.

Results

The characteristic of haploidentical activated NK cells

The apheresis was done in six haploidentical donors with 16.5*10⁹ ± 9.5*10⁹ (9.2*10⁹–35*10⁹) total nucleated cells in 324.8 ± 107.4 ml (178–488). The purity of CD56/16 positive cells was about 93.3 ± 18.5% (69.3–99.5) with mean number of 714.5*10⁶ ± 516.6*10⁶ (209*10⁶-1692*10⁶) cells (Fig. 1A, B). Isolated NK cells were expressed NKG2A (7.2%±3.8%) as inhibitory markers and NKG2D (83.0%±12.0%), NKp30 (11.6%±2.4%) and NKp46 (31.4%±13.8%) as activator markers. The inhibitory markers significantly reduce to 0.82%±0.38%, in contrast to increasing activator markers, specially NKp30 (23.7%±14.4%, P < 0.05) (Table 1, Fig. 1C, D). Interestingly, the expression pattern of activator markers was high in our six donors which may be related to Covid-19 pandemic and exposure of healthy donors to minor levels of virus, however, all of them were negative for Covid-19.

To evaluate the cytotoxic activity of activated NK cells, we co-cultured them with three types of tumors cells: K562, SKOV3, and U251 line including. Moreover, in some patients, patient-derived GBM (PD-GBM) was prepared using primary culture of tumor biopsies, to confirm the potential of NK cells to target the patients GBM cells in vitro. The results indicated the 55.0%±19.7%, 64.8%±30.1% and 43.3%±13.0 cytotoxic effects against the K562, SKOV3 and GBM lines, respectively. 75.0 ± 8.0 percent of cultivated patients-derived GBM was targeted with their haploidentical activated NK cells (Table 1, Fig. 2), (Fig. 1supplementary). The activated cells secreted more interferon gamma in cell culture media (Fig. 2).

During this study we found that although recovery of the cells reduced post thawing, but the cryopreservation did not affect the cytotoxic potential of isolated NK cells (data not shown).

In overall, all fresh and cryopreserved samples used in the present study successfully passed the quality control steps. They were negative for bacteria and fungi (Table 1). The profile of the cells prepared in this study is summarized in table1.
Based on the above results, the overall disease control rate was 33.3% (two of six cases in total).

Accordingly, the exacerbation of primary disease was found to have a clear impact on the prognosis in this case. Therefore, a causal relationship between the treatment and the outcome was established.

In cases five and six, the rapid tumor progression led to patient lost after two months (Case 5) or even sooner (Case 6). For these two cases, no postoperative imaging studies were performed, and long-term follow-up was not possible owing to early deterioration and death. Therefore, the efficacy evaluation did not show any adverse events and just two patients had the pain around two to three hours.

The clinical response of the enrolled cases is summarized in Table 2. Four patients (Case 1–4) were evaluated for the efficacy endpoint, and there was only one case with complete response without recurrence of metastatic brain tumors post 495 days (case 2). However, the evidence of carcinomatous meningitis was reported 28.2.2022, with rapid decrease in level of consciousness. She again received the 80 *10^6 cells intra thecal which caused an increase in consciousness to the normal. She is alive now with no further progression of tumor for around 1.5 years after the first enrollment in this protocol.

Safety and Preliminary efficacy.

Ten patients were screened from September 2019 to March 2021 and only six of them; 3 adults and 3 children were eligible for the present study and enrolled with a signed consent form. Four patients were deemed ineligible, due to progression of their clinical state before NK cell infusion, and delay in postoperative recovery. One patient lost follow up and in the other one the efficacy endpoint did not meet because of disease progression. At the end, only four patients reached to final analysis (Fig. 2 supplementary).

Three patients were enrolled with recurrent GBM, two patients with disseminated grade IV tumor (medulloblastoma) and one with secondary brain tumor (malignant ovarian cancer) (Table 4, supplementary). The median age was 62.8 years (range, 3–61); three patients were female. Lansky/Karnofsky performance score ranged from 70 to 100.

Four patients received three doses, the other two patients (case 6 and 1) received one and four doses of activated NK cells, respectively. The first injection was fresh, and the rests were cryopreserved. Therefore, the patients totally received 9.2x10^6 to 46.3x10^6 NK cells based on the tumor size. In those patients who had surgery for tumor resection, the NK cells were injected directly into the tumor cavity 10–15 days post-surgery. In patients with diffuse tumors who were not eligible for surgery, totally 49.3 x10^6 to 60 x10^6 NK cells were injected into the CSF two weeks after last chemotherapy.

Adverse Events (EVs) assessment was completed in a total of five patients and are summarized in Table (Table 5, supplementary). In overall, the patients did not show any adverse events and just two patients had the pain around two to three hours.

During our study and after each injection, no adverse reaction was observed, which confirmed the safety of activated haploidentical NK cells, directly injected to the tumor cavity or CSF fluid.

The clinical response of the enrolled cases is summarized in Table 2. Four patients (Case 1–4) were evaluated for the efficacy endpoint, and there was only one case with complete response without recurrence of metastatic brain tumors post 495 days (case 2). However, the evidence of carcinomatous meningitis was reported 28.2.2022, with rapid decrease in level of consciousness. She again received the 80 *10^6 cells intra thecal which caused an increase in consciousness to the normal. She is alive now with no further progression of tumor for around 1.5 years after the first enrollment in this protocol.

Three cases (cases 1, 3, 4) had Progressive disease. However, the duration of tumor growth or recurrence was different: in case 1, tumor did not growth in the site of the NK cell injection and the disease was stable for 3 months post NK infusion. Though, an MRI at 6 months of injection showed disseminated infiltrative pia-arachnoid recurrence and a new mass lesion in the right frontal lobe (Fig. 3). The patient underwent palliative chemotherapy, and the disease is stable now after 18 months of the second operation and NK therapy. The NK therapy was done for this patient again at dose 50 million cells weakly through intra thecal injection. He was alive for about 506 days post 3 doses NK therapy.

In case 3, the patient was involved with COVID-19 Infection. Meanwhile, the tumor progression was accelerated and therefore he passed away 53 days post treatment. In case 4 who had tumor residua in his last surgery, the disease was stable for about 40 days after last injection, but tumor regrowth was observed in site of tumor with vast metastasis. We lost him 168 days after his enrollment in this protocol.

In cases five and six, the rapid tumor progression led to patient lost after two months (Case 5) or even sooner (Case 6). For these two cases no postoperative imaging studies were performed, and long-term follow-up was not possible owing to early deterioration and death. Therefore, the efficacy evaluation did not perform in those patients. As the case 6 passed away within the first month of intervention, the case was discussed at Data Safety and Monitoring Board. Accordingly, the exacerbation of primary disease was found to have a clear impact on the prognosis in this case. Therefore, a causal relationship between the study protocol and the death of case 6 was rejected.

Based on the above results, the overall disease control rate was 33.3% (two of six cases in total).
Author contributions
Royan Institute for Stem Cell Biology and Technology, who support us in this study. Help us to do experiments in good manner. In particular, we acknowledge Prof. A.H. Shahverdi, head of Royan Institute and Prof. H. Baharvand, Director of Zarrabi and all staffs of Royan Stem Cell Technology Co for their interaction and collaboration, Dr. J. Firouzi, Y. Nouri, Dr. A.R shokoohifar, A.R Khazaei who helped us to do experiments in good manner.

Acknowledgment

haploidentical NK cells in malignant brain tumors is safe, feasible, tolerated at higher dose and also is cost effective. Further studies with larger sample size is demanded to assess the efficacy of this therapeutic method. At the end, our study showed that local administration of the infusions of purified NK cells of haploidentical sources using intra tumoral cavity or intrathecal injection and the primary objective of feasibility and safety was achieved. However, the allogenic NK cells from peripheral or cord blood could more proper, as are highly cytotoxic and cause minimal risk of GvHD[22]. Haploidentical NK cells classically is used in blood malignant disorders[25]. However, there is not any reports in subject of haploidentical NK cell therapy in GBM or malignant brain tumors. Given the local route of delivery of haploidentical NK cells and the possibility of an adverse immunological response, we started with multiple infusions of purified NK cells of haploidentical sources using intra tumoral cavity or intrathecal injection and the primary objective of feasibility and safety was achieved. Although the number of patients was low in the present study and was contain both pediatric and adult patients, but we achieved the secondary objective in two patients with increasing their overall survival. No side effects were seen in patients with increasing in the quality of life. The clinical symptom, such as their behavior including walking and swallowing, improved in all patients shortly post three NK cell infusion. Meanwhile, the disease progression was observed in most of patients, as were in end stage of disease. We found that some criteria may affect the secondary objectives of NK cell therapy in glioblastoma patients. In first, the time of NK cell infusion is really important. It seems that in those patients (Case 1, 2) that NK cell therapy straight forwardly was done after surgery and before chemo/radiotherapy, the better outcome with increasing in overall survival was observed. Secondly, the number of cells and infusion's interval also affect the outcome of the patients. Here we injected maximum 80x10^6 million cells that was tolerated for patients, three times weekly. However, the injections should be continued in the good responder patients, who had improvement in their clinical symptom or their quality of life. Thirdly, tumor residual or remnant tumor mass post-surgery or tumor regrowth post radio/chemotherapy may reduce the affect the NK therapy. The patients 3–6 who had remnant tumor and was resistance to chemo/radio therapy, they are not good responder to our treatment protocol. We assumed that if they received the NK cells not at the end stage of their disease but sooner at the starting phase of treatment may have this chance to increase their overall survival. Interestingly, patients post treatment follow up and MRI results demonstrated that there was not any tumor recurrence at the previous tumor bed site and if there was tumor relapse in the months following cell therapy, it was seen in an area other than the previous tumor site. Thus, we suggested in patient with possibility of surgery, the NK infusion could be done at tumor surgery cavity and intrathecal one by one to reduce tumor regrowth and tumor metastasis with providing NK cells in CSF circulation. Even though, the number of cases in the present study are not enough to draw such a conclusion, and further studies with larger sample size is demanded to assess the efficacy of this therapeutic method. At the end, our study showed that local administration of the haploidentical NK cells in malignant brain tumors is safe, feasible, tolerated at higher dose and also is cost effective.

Table 2. Preliminary Clinical Response

<table>
<thead>
<tr>
<th>Patient#</th>
<th>No. of Administration</th>
<th>Total Cell Administration (*10^6)</th>
<th>Best overall response</th>
<th>PFS (Days)</th>
<th>OS (Days)/Status*</th>
</tr>
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<tbody>
<tr>
<td>1</td>
<td>4</td>
<td>9.2</td>
<td>PD</td>
<td>90</td>
<td>506/A</td>
</tr>
<tr>
<td>2</td>
<td>3</td>
<td>9.6</td>
<td>CR</td>
<td>495</td>
<td>495/A</td>
</tr>
<tr>
<td>3</td>
<td>3</td>
<td>18</td>
<td>PD</td>
<td>-</td>
<td>53/D</td>
</tr>
<tr>
<td>4</td>
<td>3</td>
<td>46.3</td>
<td>PD</td>
<td>-</td>
<td>168/D</td>
</tr>
<tr>
<td>5</td>
<td>3</td>
<td>49.3</td>
<td>PD</td>
<td>-</td>
<td>90/D</td>
</tr>
<tr>
<td>6</td>
<td>1</td>
<td>60</td>
<td>PD</td>
<td>-</td>
<td>2/D</td>
</tr>
</tbody>
</table>

A: Alive, CR: Complete Response, D: Dead, OS: Overall Survival; PD: Progressive Disease; PFS: Progression Free survival; SD: Stable Disease
*At study endpoint

Discussion

The results of our previous studies proved the effectiveness of the intra tumor injection of NK cells in elimination of GBM cells in the animal model [14]. Therefore, the safety and feasibility of repeated injection of activated haploidential NK cells into tumor bed side or using intra thecal injection was studied. Typically, the large number of NK cells (10^7cells/kg to 4.7×10^10 total NK) has been administrated as intra-venous injection (IV Route) [22] in brain tumor patients; however the cell trafficking and their transmission from blood brain barrier under question. Moreover, most of the injected NK cells lost because of their trapping in kidney, spleen, lung and consequently small number of activated cells may entrance to tumor mass. Intra thecal and intraventricular infusion are another route for NK cell therapy in brain tumors[23], which make close immune cells to target cells and also reduce cell number in each injection which make cost effective the NK therapy. Choosing the NK cell source is another drawback that should be noticed in immune cell therapy in different cancers. In brain tumors and GBM patients, autologous NK cells is used to reduce the unwanted allogenic reaction[24], however exert limited cytotoxicity against autologous tumors[22].

However, the allogenic NK cells from peripheral or cord blood could more proper, as are highly cytotoxic and cause minimal risk of GvHD[22]. Haploidentical NK cells classically is used in blood malignant disorders[25]. However, there is not any reports in subject of haploidentical NK cell therapy in GBM or malignant brain tumors. Given the local route of delivery of haploidentical NK cells and the possibility of an adverse immunological response, we started with multiple infusions of purified NK cells of haploidentical sources using intra tumoral cavity or intrathecal injection and the primary objective of feasibility and safety was achieved. Although the number of patients was low in the present study and was contain both pediatric and adult patients, but we achieved the secondary objective in two patients with increasing their overall survival. No side effects were seen in patients with increasing in the quality of life. The clinical symptom, such as their behavior including walking and swallowing, improved in all patients shortly post three NK cell infusion. Meanwhile, the disease progression was observed in most of patients, as were in end stage of disease. We found that some criteria may affect the secondary objectives of NK cell therapy in glioblastoma patients. In first, the time of NK cell infusion is really important. It seems that in those patients (Case 1, 2) that NK cell therapy straight forwardly was done after surgery and before chemo/radiotherapy, the better outcome with increasing in overall survival was observed. Secondly, the number of cells and infusion's interval also affect the outcome of the patients. Here we injected maximum 80x10^6 million cells that was tolerated for patients, three times weekly. However, the injections should be continued in the good responder patients, who had improvement in their clinical symptom or their quality of life. Thirdly, tumor residual or remnant tumor mass post-surgery or tumor regrowth post radio/chemotherapy may reduce the affect the NK therapy. The patients 3–6 who had remnant tumor and was resistance to chemo/radio therapy, they are not good responder to our treatment protocol. We assumed that if they received the NK cells not at the end stage of their disease but sooner at the starting phase of treatment may have this chance to increase their overall survival. Interestingly, patients post treatment follow up and MRI results demonstrated that there was not any tumor recurrence at the previous tumor bed site and if there was tumor relapse in the months following cell therapy, it was seen in an area other than the previous tumor site. Thus, we suggested in patient with possibility of surgery, the NK infusion could be done at tumor surgery cavity and intrathecal one by one to reduce tumor regrowth and tumor metastasis with providing NK cells in CSF circulation. Even though, the number of cases in the present study are not enough to draw such a conclusion, and further studies with larger sample size is demanded to assess the efficacy of this therapeutic method. At the end, our study showed that local administration of the haploidentical NK cells in malignant brain tumors is safe, feasible, tolerated at higher dose and also is cost effective.

Declarations

Acknowledgment

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Author contributions

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Study conception and supervising by M.E, A.H, and Z.H. project administration, study design, and management by N.S.A. Donor selection by M.B and R.M. Clinical intervention by Z.H, R.S, and M.F. Data collection by N.S.A and M.E. Flowcytometry by M.A. Cell isolation by N.S.A. and A.I. First manuscript draft written by Y.J, N.S.A, and M.E. All authors reviewed, edited, and approved the final manuscript.

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Conflict of Interest

The authors have no relevant financial or non-financial interest to disclose.

Ethical approval

This clinical trial study was approved by Royan ethical committee, iranian registry of clinical trials, and international registry of clinical trials.

Consent for participate

Informed consent of all participants (patients and donors) was obtained.

References

Supplementary

The Graphical Abstract is not provided with this version.

Figures

**Figure 1**

The characterization of purified NK cells. (A) The percentage of NK, NKT, T cells pre and post purification in one selected donor. (B) The bar-graph of purified cells in six different healthy donors. The data was shown as Mean± SD, ** P<0.01*** , P<0.001 (C) The percentage of activator and inhibitor markers expressed on purified NK cells. The data was shown as Mean± SD, * P<0.05 (D) The histogram flowcytometry data from one selected donor before and after activation using IL-15.

**Figure 2**
The Cytotoxic effects of NK cells (inactive/active) on different type of cancer cells including K562, SKOV3, U251 and PD-GBM and the interferon gamma release assay. (A) Flowcytometry graphs for selected samples (first and second row). (B) The cytotoxicity potential of all purified NK cells. Data shows as Mean±SD of six purified NK cells. (C) The activated NK cells can release IFN-γ more than inactive ones in exposure to 3 types of tumor cells. (D) The mean concentration of released IFN-γ via active and inactive NK cells.

Figure 3

The MR images of four patients who received the NK cells and followed up based on the approved protocol in the present study. The first row of pictures shows the schematic of cell infusion in patients who had tumor surgery. In these patients NK cells infused exactly in site of surgery post healing the lesions (Patients 1, 3-4). The patients 2 received NK cells as intra thecal infusion.

Supplementary Files

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- supplementaryofarticle.docx