Oncolytic Virus Ad-TD-nsIL12 for Primary Pediatric Diffuse Intrinsic Pontine Glioma

Research Protocol

Investigator protocol signature page

I have read the protocol, including all appendices, and agree to conduct this study in accordance with the current protocol.

Hongwei Zhang _____

Principal investigator Signature

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Date:

Contents

1. Basic information

1.1. Purpose of the study

- Main objective
  To observe the safety, tolerability and toxicity of Ad-TD-nsIL12 intratumoral injection in pediatric DIPG patients (NCI-CTCAE V5.0).
- Secondary objectives
  - 12-month overall survival (OS12), tumor response, Ad-TD-nsIL12-induced immune response.
  - Change in quality of life over time.
  - Collect tumor and blood samples for molecular and immunological studies.

1.2. Foundation Information

Supported by the National Key Research and Development Program of China (No. 2019YFC1316104): Clinical trials and therapeutic mechanisms of novel oncolytic viruses for tumor therapy.

1.3. Study location

Sanbo Brain Hospital, Capital Medical University, Beijing, China.

1.4. Research time

From January 2023, the estimated research period is 2 years. Enrollment of the first patient is expected to occur on January 4th, 2023.

1.5. Study diseases

Primary Pediatric Diffuse Intrinsic Pontine Glioma (DIPG).

1.6. Estimated number of patients

9-18 people.

2. Background and feasibility of the study

2.1. Background

Diffuse intrinsic pontine glioma (DIPG) is a disease associated with histone mutations and is the leading cause of brain tumor-related death in children. Due to the specificity of the tumor location and the high infiltration, the current treatment options for this disease are very limited. Despite of the short-term benefit of standardized radiotherapy in the treatment of DIPG, the prognosis remains poor, with progressive neurological deterioration and median survival less than 1 year and 2-year survival less than 10%. Due to the severe lack of treatment for DIPG, it is imperative to seek a variety of emerging treatments, including oncolytic viruses.

Over the past 20 years, molecular and genetic tools have been available to build new viruses that transfer exogenous genes into cancer cells. The initial study used viral vectors with replication defects to deliver various genes to target tissues. These gene therapies have also shown efficacy and safety in multiple preclinical tumor animal models. However, these promising preclinical results have not been successfully translated into patient benefit in clinical practice. This defect is largely due to the inability of viral vectors to transform cancer cells in large numbers. Typically, these vectors infect only a small number of cells in the body, and in the best case no more than 15%. With the development of molecular biology and genetic engineering technology, viruses selective replication in tumor cells became possible, leading to tumor infection and continuous tumor destruction. This method was first developed and utilized in herpesviruses. In recent years, many other kinds of oncolytic viruses with selective ability to replicate in tumor have been developed. Adenoviruses (AdV) are particularly well-suited to this approach because they cause the least disease in humans and can be genetically

manipulated to infect and replicate in dividing and non-dividing cells. A variety of highly replicable AdV has been shown to be safe in clinical trials. E1B-55K and E3B gene deletion adenovirus type 5 (Ad5) H101 is the first international oncolytic virus product approved by China in 2005. Similarly, recombinant replicating active AdV, containing multiple gene deletions, has been produced, and their effectiveness as therapeutic agents has been validated in vivo and in vivo models of many primary and metastatic tumors. These animal model-based experiments provide valuable information about the effectiveness and safety of the virus. Experiments have clearly demonstrated that by altering these genes, whether through deletion, insertion, or point mutation in coding regions, the resulting virus will kill dividing tumor cells but is non-toxic after delivery to normal brain tissue.

Therefore, it is possible to develop an ideal, stable genetically engineered virus to selectively kill glioma cells. The brain is an immune "exempt" area, which is also a reason for the poor treatment effect, rapid development and poor prognosis of brain tumors, which is an advantage of oncolytic virus therapy. Therefore, brain tumors are suitable targets for intervention by oncolytic viruses, which use conditionally replicated viruses to replicate in tumors and kill tumor cells while preserving the normal cell population in the brain. In recent years, some clinical trials of oncolytic viruses for the treatment of brain tumors have shown good treatment prospects.

2.2. Pre-clinical Research on Ad-TD-nsIL12

In terms of safety, all preclinical safety studies of Ad-TD-nsIL12 have been completed, and the long-term toxicity tests of golden gopher and cynomolgus monkeys indicate that the safe dose of Ad-TD-nsIL12 is at the level of 5×10^{11}vp, which is significantly higher than the effective dose of tumor lysis in animal tests. In terms of efficacy, completed preclinical pharmacodynamic studies of Ad-TD-nsIL12 have shown that Ad-TD-nsIL12 at 5×10^{9} vp levels can effectively treat a variety of solid tumor models in nude mice and golden gopher. At the same time, the production process of Ad-TD-nsIL12 is clear and controllable, and the quality of the three batches of pilot continuous production is all qualified and stable.

2.3. A study of Ad-TD-nsIL12 in the treatment of recurrent glioblastoma

Between December 2019 and September 2022, a total of 8 patients were recruited to receive Ad-TD-nsIL12 treatment at the Sanbo Brain Hospital of Capital Medical University. The trial assigned three dose-escalation cohorts ($5x10^{9}$, $1x10^{10,}$ $5x10^{10}$vp, 1ml). The most common adverse events were fever and cognitive impairment. Nausea and vomiting also occurred frequently, and the dose used in Cohort 2 ($1x10^{10}$vp) was used as the maximum tolerated dose (MTD).

One patient had a complete response after treatment with Ad-TD-nsIL12 and is still alive, while another patient received a partial response. In addition, Ad-TD-nsIL12-related proteins (EIA and hexon) were observed in both post-treatment samples, indicating that Ad-TD-nsIL12 remained active for a longer period of time after surgery. Clearance of the enhancement in the injection area was observed after administration of Ad-TD-nsIL12, and the samples collected also showed higher infiltration of $CD3^{+}$, $CD4^{+}$ and $CD8^{+}$ cells than pre-treatment samples. This is consistent with preclinical studies: the antitumor efficacy of Ad-TD-nsIL12 depends on $CD8^{+}$ T cells and suggests that Ad-TD-nsIL12 induces a long-term immune response.

3. Screening of study patients

3.1. Inclusion criteria

3.1.1. Informed consent of the parents or patient.

3.1.2. Patient must be, in the investigator opinion, able to comply with all the protocol procedures.
3.1.3. Age 1-18 years.
3.1.4. A negative pregnancy test in fertile women (women are considered of childbearing potential (WOCBP) after menarche, unless permanently infertile, including hysterectomy, bilateral salpingectomy, and bilateral oophorectomy).
3.1.5. Patient newly diagnosed of DIPG in MRI.
3.1.6. Pre-enrollment patients LPS (patients aged ≥1 and <16 years) and KPS (patients aged ≥16 years) ≥ 50.
3.1.7. Lesion considered by the investigator to be accessible for stereotactic biopsy. The location of the lesion allows injection without virus entering the ventricular system.
3.1.8. No previous treatment for DIPG.

3.2. Exclusion criteria

3.2.1. Serious infections or intercurrent conditions, including but not limited to severe renal failure, liver failure, heart failure, or bone marrow failure, which are not permitted for inclusion according to the investigator's criteria. Patients must be afebrile at baseline (<38°C).
3.2.2. Other investigational medications within 30 days prior to viral treatment.
3.2.3. Participants with immunodeficiency, autoimmune disease, or active hepatitis.
3.2.4. Any medical or psychological condition that might interfere with the patient's ability to participate if older than 16 years or parents ability when younger than 16, or give informed consent or would compromise the patient's ability to tolerate therapy or any disease that will obscure toxicity or dangerously alter drug metabolism.
3.2.5. Tumor with multiple location.
3.2.6. Pregnant or breast-feeding females.
3.2.7. Severe bone marrow hypoplasia.
3.2.8. Transaminases (aspartate aminotransferase (AST) and/or alanine aminotransferase (ALT)) or total bilirubin > 3 times the upper limit of normal.
3.2.9. Neutrophils < $1x10^9$/L.
3.2.10. Platelets ≤ $100x10^9$/L.
3.2.11. Hemoglobin < 9 g/dl.
3.2.12. Patients with Li-Fraumini syndrome or a known germline defect in the retinoblastoma gene or its associated pathways.
3.2.13. Administer any type of vaccine within 30 days prior to Ad-TD-nsIL12 administration.
3.2.14. Blood transfusions or drugs (such as G-CSF) within 28 days before baseline to treat pancytopenia or other blood disorders.

4. Study design

4.1. Overall design

Single-arm, single-center, drug safety assessment clinical trial with a 3+3 dose escalation design.

4.2. Screening and Selections

Patients will be screened at the clinic by the investigators. Screening will be performed within 15 days prior to the use of Ad-TD-nsIL12. Prior to screening, investigators will present parents and patients with complete information about the clinical study and sign an informed consent form. Enrollment to the trial will be possible only if the following screening trials are performed and the inclusion and exclusion criteria are met:

- Physical examination, including vital signs and weight.
- Neurological examination and functional status assessment (including Lansky

Performance Status Score (LPS) for patients aged one to under 16 years of age, and Karnofsky Performance Status (KPS) for patients 16 years of age or older).

- Quality of Life Assessment (PedsQL^{TM}).
- Clinical laboratory tests, including hematology, chemistry (containing C-reactive protein), and coagulation.
- Detection of adenovirus antigens and anti-adenovirus antibodies in serum.
- Pregnancy test (serum).
- Serological testing for HIV and HBV/HCV.
- MRI (with or without gadolinium), baseline (completed within 15 days prior to viral treatment).
- Medication.

To reduce losing follow-up, participants will be asked to provide contact details of two or more relatives.

4.3. In-hospital period

- After stereotactic biopsy, the Ommaya reservoir will be inserted through the biopsy channel and a single injection of Ad-TD-nsIL12 will be delivered immediately after surgery.
- The injection dose start with $3x10^9$vp (D1) and follow $1x10^{10}$vp (D2), $3x10^{10}$vp (D3) for climbing. If 2 or 3 patients develop severe adverse event (SAE) according to NCI-CTCAE at D1. The dose will then be reduced to $1x10^9$vp (D0). If one patient develops SAE at the D1 dose, the other 3 patients will continue to be included with the D1 dose to assess whether the occurrence of this SAE is accidental. If SAE develops in 1 of the next 3 patients, following patients will receive the D0 dose.
- Before subsequent patients enrollment, the former patient in each cohort must be observed for at least 6 days after virus injection. The administration of virus to participants in the new cohort will be at least 10 days behind the last participant in the previous cohort.
- If the patient presents with symptoms of decreased brain function or decreased cardiovascular perfusion, or if there is an allergic reaction or signs of anaphylaxis, administration of Ad-TD-nsIL12 should be discontinued. In addition, treatment of Ad-TD-nsIL12 will also be interrupted if other AEs deemed necessary by investigators to be interrupted. In addition, if ventricular penetration is considered to have occurred during viral use, the dosing procedure will be terminated and no further treatment with Ad-TD-nsIL12 will be given, and such patients will be observed and examined by virologic testing (AdV) and further follow-up.
- The tumor tissue obtained at the time of biopsy or excision, as well as the collected blood samples, will be archived in our hospital for future analysis.
- Visit 0: 3±1 days after Ad-TD-nsIL12 injection. Including:
  - Physical examination, including vital signs, weight, and height;
  - Neurological examination and functional status assessment (LPS or KPS), life quality assessment;
  - Blood tests, blood biochemistry (containing C-reactive protein), coagulation function assessment and other blood tests (including detection of adenovirus antigens and anti-adenovirus antibodies in serum);
  - MRI (with or without gadolinium) and tumor response assessment;
  - Assessment and documentation of adverse events, including concomitant medications, including steroids.
- When the patient is discharged, the patient and his or her relatives will be provided with the investigator's contact information in case of an emergency. In addition,

after discharge, the patient can follow the recommended treatment of his/her pediatric oncologist in other hospitals, and the investigators of the experimental group will contact the hospital for follow-up visits. If the investigators think it is necessary, follow-up could be done at Sanbo Brain Hospital.

- Radiation therapy will start 2-6 weeks after the biopsy and virus injection at the discretion of the radiation therapist.

4.4. Follow-up period

- After enrollment, clinical follow-up will be conducted every 4 weeks for the first 3 months after viral treatment. After 3 months, follow-up visits can be performed by phone, images and blood tests results can be sent by email, or patients can come to our hospital for treatment and follow-up if they wish. Treatment tolerability was measured by severity classification of adverse events (NCI-CTCAE V5.0) and correlation with the investigating drug.
- Visit 1: 28±5 days after Ad-TD-nsIL12 injection. Including:
  - Physical examination, including vital signs, weight, and height.
  - Neurological examination and functional status assessment (LPS or KPS);
  - Quality of Life Assessment (PedsQL^{TM});
  - Laboratory blood tests, some of which will be retained for subsequent analysis;
  - MRI (with or without gadolinium) and tumor response assessment;
  - Evaluation and documentation of adverse events, concomitant medications;
  - Steroid evaluation.
- Visit 2: 8±1 weeks after Ad-TD-nsIL12 injection. Including:
  - Physical examination, including vital signs, weight, and height.
  - Neurological examination and functional status assessment (LPS or KPS);
  - Quality of Life Assessment (PedsQL$^{TM)}$;
  - Laboratory blood tests, some of which will be retained for subsequent analysis;
  - MRI (with or without gadolinium) and tumor response assessment;
  - Evaluation and documentation of adverse events, concomitant medications;
  - Steroid evaluation.
- Visit 3: 12±1 weeks after Ad-TD-nsIL12 injection. Including:
  - Physical examination, including vital signs, weight, and height.
  - Neurological examination and functional status assessment (LPS or KPS);
  - Quality of Life Assessment (PedsQL^{TM});
  - Laboratory blood tests, some of which will be retained for subsequent analysis;
  - MRI (with or without gadolinium) and tumor response assessment;
  - Evaluation and documentation of adverse events, concomitant medications;
  - Steroid evaluation.
- Visit 3 is the last designated clinical follow-up, after which the patient's overall AE will be concluded and assessed.
- Except for the 3 planned visits, both patients and investigators may request unplanned follow-up at any time.
- During follow-up, if viral infection of CSF is suspected, the investigator will decide whether to perform virological (AdV) testing.

4.5. Remote follow-up

- Remote follow-up contact: (6 months ± 1 month, 12 months ± 1 month, 18 months ± 1 month, 24 months ± 1 month)
- Visit :
  - Survival;
  - Assessment of quality of life;

- Evaluation and documentation of adverse events, concomitant medications;
- Steroid use;
- If any imaging tests are performed, radiological information is collected and analyzed.

- After that, patients will continue to receive standardized treatment for DIPG, and investigators will continue to follow up on their basic information, including but not limited to survival, quality of life, and occurrence of AE. Depending on the special and necessary clinical situation, the investigator may request increased follow-up frequency, or even follow-up in Sanbo Brain Hospital.
- At the time of tumor progression, the study will end, the patient and their guardians could chose following treatment, and the investigators will continue to collect basic information about clinical status (including at least survival and quality of life assessment) until the patient dies or at least 2 years after virus injection.
- If the tumor does not progress, the investigators will continue to collect basic information about clinical status (including at least survival and quality of life assessment) until the patient dies or at least 5 years after virus injection. Again, researchers can decide to conduct a final analysis of the study data or to continue long-term follow-up of certain patients at any time, this decision that can only be made after all patients have died or been followed for more than 1 year.

4.6. Follow-up of laboratory tests

Basic blood tests will be done before and early (within 3 days) after virus injection, and during 1 to 3 visits. These tests will include, but are not limited to:

- Hematology: white blood cell count with differential, including neutrophils, lymphocytes, monocytes, eosinophils and basophils, red blood cell count, hemoglobin, hematocrit, platelets, mean platelet volume (MPV), MCV, MCH, MCHC, RDW.
- Chemical and C-reactive proteins: glucose (fasting), creatinine, sodium, potassium, chloride, calcium and C-reactive proteins.
- Coagulation: PT, APTT

4.7. Radiological follow-up

- MRI will be used to evaluate tumor response, and assessment of tumor reflection requires a uniform sequence from baseline (T2 or enhanced phase recommended).
- Time point
  - Within 15 days before Ad-TD-nsIL12 injection (baseline);
  - Within 3 days after Ad-TD-nsIL12 injection;
  - Visit 1 (28±5 days after Ad-TD-nsIL12 injection);
  - Visit 2 (8±1 weeks after Ad-TD-nsIL12 injection);
  - Visit 3 (12±1 weeks after Ad-TD-nsIL12 injection).
- The RAPNO criteria (2020) will be used to assess tumor response. If a pseudoprogression is suspected according to image changes within the first two months after viral injection, the final verdict will be made by a radiologist based on the advanced MRI sequence. After the final planned MRI is completed (Visti 3), the study procedure will be completed, after which the patient will continue to receive standardized treatment, and the investigator will continue to follow up with basic information, including but not limited to survival, quality of life, and occurrence of AE.
- Researchers can request an MRI at any time during the study to determine the imaging response of the tumor.

4.8. Dose escalation and determination of the maximum tolerated dose

(MTD).

- The dose will start with $3x10^9$vp. Three dose stages are planned: $3x10^9$vp, $1x10^{10}$vp as well $3x10^{10}$vp。
- Each cohort will include 3 to 6 participants depending on the occurrence of AE. The criteria for dose climbing are as follows:
  - If there were no Ad-TD-nsIL12 related SAEs (NCI-CTCAE ≥grade 3) occurred within 6 days after virus injection in the previous patient in the same cohort, the latter patient continues to be included.
  - If 1 patient developed Ad-TD-nsIL12 related SAE in a cohort, 3 patients will continue to be included in this cohort to verify whether it's accidental, and if any 1 of the subsequent 3 patients developed Ad-TD-nsIL12 related SAE, it would be considered as the dose limiting toxicity (DLT), and the previous dose is taken as the maximum tolerated dose (MTD).
  - If 2 patients in the same cohort developed investigational drug-related SAE, it will be considered to be DLT, then the trial will be terminated and the dose used in previous cohort will be defined as MTD.
  - If all 3 patients in the same cohort had no drug-related SAE within 10 days after virus injection, the dose would be upgraded to the next cohort.
  - If DLT presented in the first cohort (D1), dose adjustment will be made according to what is described in protocol 4.3, and if DLT still occurs at D0, the trial terminates.
  - If DLT still does not occur at the highest dose D3, MTD is not defined and the trial terminates.
- Any clinical SAEs deemed to be likely to be associated with Ad-TD-nsIL12 will be recorded by the investigator and graded accordingly. Follow-up will continue until the SAE disappears or drops to grade 1.
- In addition, if non-neurological complications related to any surgical or anesthesia management occur (including, but not limited to, respiratory failure, pneumonia and other systemic infections, cardiac arrest, myocardial infarction, pulmonary embolism, deep vein thrombosis, tracheal and laryngeal trauma, postanesthesia lethargy, fatigue, and short-term memory loss), dose reduction may be considered after discussion with the principal investigator.

4.9. Withdrawal from the study/discontinuation of participants

Patients can withdraw from the study at any time without explanation. The investigators will try to collate and analyze clinical data at the time of withdrawal. Patients who withdraw will not be replaced, and investigators will continue to record their survival and test tissue samples obtained prior to withdrawal. Patients who complete an investigational drug-related AE assessment prior to withdrawal from the study will be included in the safety assessment of Ad-TD-nsIL12 as a valid case.

4.10. Duration of participation of each patient

Each patient will receive viral injections prior to any other oncology treatment and will undergo clinical and radiological follow-up within 3 months after Ad-TD-nsIL12 treatment. Then, patients will continue to be followed up until two years. The research team will contact each patient by phone or mail at 6, 12, 18 and 24 months to update clinical information. Patients will continue to receive follow-up until five years if they are still alive at 24 months after viral treatment. At any time, patients can freely withdraw.

5. Experimental drug

5.1. Drug introduction

Ad-TD-nsIL12 is an adenovirus that makes the virus replicate only in tumor cells by deleting E1A-CR2, enhancing targeting and reducing toxicity to normal cells. Deletion of E1B-19K accelerates apoptosis of infected tumor cells and enhances the ability to kill tumor cells. Deletion of E3-gp19K is beneficial to tumor-specific antigen presentation in cells and enhances anti-tumor effect. Retain E1B-55K to ensure the replication ability of the virus and improve the tumor killing ability. Preserves E3B, inhibits macrophage-mediated viral clearance, improves antitumor effect. In addition, IL-12 is introduced into Ad-TD to realize tumor immunogene therapy and further improve the effect of tumor treatment. After modification, the appropriate level of IL-12 in the blood can be maintained, so that the systemic anti-tumor effect of IL-12 is stronger, and the side effects of high concentrations of IL-12 in the blood are also avoided.

5.2. Formulation and storage

Formulation: The final Ad-TD-nsIL12 product is BioTTT001, formulated with a special virus protection solution. The concentration of reagents was 5×10^{11} vp/mL at 1 mL each. Storage: Ad-TD-nsIL12 vials should be stored in the research pharmacy freezer at ≤-80°C prior to dilution. The diluent should be kept at room temperature. All research supplies will be kept in a properly locked room where only pharmacy staff will be allowed to enter and the temperature of the freezer must be monitored regularly.

5.3. Stability testing

Ad-TD-nsIL12 has passed the stability test of biological products, which has good stability and a shelf life of up to two years. Stability-tested properties of final biologics include appearance, potency (viral titer, viral particle concentration, vp/IU ratio), and safety. Virus appearance, titer, viral particle concentration, and vp/IU ratio were detected at 0, 3, 6, 12, 24, and 36 months time points. The products of the three batches of pilot continuous production are all qualified and stable.

5.4. Production establishment

It is entrusted by Beijing Hammer Biotechnology Co., Ltd. to Shenzhen Yuanxing Gene Technology Co., Ltd. to produce.

5.5. Handlers

The handling and preparation of Ad-TD-nsIL12 will follow the institutional standards for biosafety secondary reagents as specified in the Hazards and Biosafety Manual and Biosafety in Microbiology and Biomedical Laboratories. In addition, standard chemotherapy preparation precautions (gowns, gloves, masks, and glasses) and aseptic techniques will be followed when preparing the required dose of Ad-TD-nsIL12. Only needle-locked syringes or disposable syringes are used to inject or inhale biohazardous liquids. Caution should be used when handling needles and syringes to avoid self-spraying and aerosol generation during use and disposal. The original Ad-TD-nsIL12 vial and all syringes, needles, and needle caps that come into contact with Ad-TD-nsIL12 should be placed in a disinfectant cup after the procedure.

6. Response assessment

6.1. Primary assessment

- Adverse events (according to NCI-CTCAE v 5.0)
- Neurological examination
- LPS or KPS score
- Laboratory test results
- Use of concomitant drugs

6.2. Secondary assessment

- Efficacy will be assessed based on survival and tumor response as determined by

RAPNO criteria. Time to disease progression, 6-month progression-free survival, median progression-free survival, 12-month overall survival (OS12), and median overall survival will also be determined. Quality of Life Assessment (PedsQL^{TM}). Ad-TD-nsIL12-induced immune response.

7. Risks and management associated with Ad-TD-nsIL12 therapy

7.1. Potential risks

Ad-TD-nsIL12 can cause, or is likely to cause, the following conditions:

- Systemic viral infections, such as flu-like symptoms (chills, stiffness, myalgia, arthralgia, lymphadenopathy).
- Allergic reactions, such as itching, hives, changes in blood pressure, or respiratory infections that make it difficult to breathe.
- Brain infections.
- Hepatitis, which may present with abdominal pain, jaundice, abnormal liver function tests.

Ad-TD-nsIL12 has been carefully designed to reduce its ability to cause toxic effects, but side effects are still possible. If there are signs of neurotoxicity associated with Ad-TD-nsIL12 infection, supportive care and medical treatment will be implemented as needed. Neurotoxic effects, if they occur, may be short-term. However, permanent damage is also theoretically possible. As with any clinical trial, unforeseen risks are possible. In China, many people have been exposed to AdV before and already have antibodies. If patients treated with Ad-TD-nsIL12 have never been exposed to AdV before, they may develop antibodies during the study. Although the effects of wild-type AdV on the developing embryo or fetus are known and may cause miscarriage, fetal death, fetal infection, or developmental abnormalities, there are no data on the effects of Ad-TD-nsIL12 in utero. Ad-TD-nsIL12 may enter the fetus through the placenta. Therefore, all patients and their partners are advised to use appropriate forms of contraception during their participation in this study and for 6 months after administration of Ad-TD-nsIL12. Women suspected of pregnancy during the study and needed to be checked for pregnancy status at any time.

7.2. Treatment of severe AdV infection

Confirmation of the presence of AdV infection is important for deciding on antiviral agents, ruling out other treatable infections, determining prognosis, and initiating infection control measures if appropriate. If serious AdV infection is suspected after administration of Ad-TD-nsIL12, laboratory investigations such as polymerase chain reaction (PCR) and culture, analysis of cerebrospinal fluid and serum to rule out AdV infection will be performed. Other diagnostic tests such as antigen measurement, serotyping, and serology may be indicated. Additional MRI scans and brain biopsies may also be needed to determine the presence of AdV. Ribavirin, ganciclovir, cidofovir, immunotherapy, or other antiviral therapy can be started immediately, followed by appropriate treatment of the patient.

7.3. Treatment-related risks

- Biopsy

  Prior to injection of Ad-TD-nsIL12, frozen sections of the tumor will be obtained to confirm the diagnosis of DIPG. If encephalitis is suspected, stereotactic biopsy may be performed to assess the presence of Ad-TD-nsIL12 or wild-type AdV infection or, if necessary, to confirm tumor progression. Risks of brain biopsy include hemorrhage, hematoma, infection (local infection, brain abscess, meningitis), fever, pain, increased intracranial pressure, general decline in consciousness, focal neurologic deficits (e.g.,

weakness, loss of sensation, speech disturbance, aphasia, memory loss, loss of vision, diplopia, loss of cognitive function), seizures, stroke, coma, and death.

- Tumor resection
  The risks associated with craniotomy and tumor removal are varied, including but not limited to pain, hemorrhage, hematoma, epilepsy, stroke, infection, fever, decreased level of consciousness, focal neurological deficits (eg, weakness, sensory loss, speech impairment, aphasia, memory loss, vision loss, diplopia, and any other deficit of brain function, complete or partial loss of cognitive function), coma, and death. In addition, there are non-neurologic risks that can arise from any surgery or anesthesia (including, but not limited to, respiratory failure, pneumonia, cardiac arrest, myocardial infarction, pulmonary embolism, deep vein thrombosis).
- MRI
  Risks associated with MRI include claustrophobia and anxiety reactions. Reactions to the contrast medium gadolinium include; Nausea, headache, hot flashes, and palpitations. Allergic reactions to gadolinium include: skin rash, urticaria, dyspnea, in extreme cases, anaphylactic shock and possible death. Individuals with any contraindications to MRI (e.g., pacemakers, epicardial pacemaker lines, infusion pumps, surgical and/or aneurysm clips, shrapnel, metal prostheses, potentially magnetic implants, metal objects in the eye, etc.) will not be included. In addition, individuals with tumors that cannot be assessed by MRI will not be eligible for the study.
- Encephalitis
  Postoperative fever is not uncommon. However, fever of ≥ 38.9°C (with or without seizures) lasting ≥ 48 hours, decreased neurologic status and increased areas of hemorrhagic necrosis outside the tumor boundary on MRI, may indicate viral encephalitis. When appropriate, stereotactic biopsy should be performed to determine the presence of Ad-TD-nsIL12 or AdV (wild-type). The procedure of a biopsy involves taking a sample of tumor tissue through a biopsy needle and sending it to a hospital laboratory for testing. Biopsy specimens undergo standard hematoxylin and eosin (H&E) staining, as well as immunostaining. In addition, if the specimen is sufficient, tests such as PCR and culture to detect AdV and Ad-TD-nsIL12 will be performed. The pathologist will review the processed biopsy tissue. Antiviral therapy is given as appropriate.
- Focal neurological deficits
  Decreased neurological status (eg, decreased mental status), including focal neurologic deficits or specific neurologic deficits (paralysis, speech impairment, depending on tumor location), can be attributed to tumor progression, edema, hemorrhage, hydrocephalus, or encephalitis. CT and, if appropriate, MRI to help determine the cause of these neurological changes.
- Hemorrhage
  PT/INR, PTT, and platelet count are measured. Small hematomas can be treated conservatively. Large haematomas or hematomas associated with progressive neurological deterioration (i.e. > grade 3) may require surgical hematoma removal and decompression. The acceptable degree of neurotoxicity secondary to hematoma is (CTC < grade 3).
- Increased intracranial pressure

Symptoms in patients with mass masses may be related to increased intracranial pressure. Symptoms may worsen, especially after an injection of Ad-TD-nsIL12. For more extreme cases, standard treatment includes dexamethasone or mannitol.

- Necrotizing meningitis
  Response to Ad-TD-nsIL12 may cause inflammation or meningeoid symptoms. Symptoms may be self-limited and controlled with steroids and/or analgesics, but in some cases may be severe enough to cause nerve damage or death. To exclude infection, it may be necessary to perform a lumbar puncture.
- Cerebral edema
  Cerebral edema usually occurs postoperatively in patients with tumors and usually requires standard measures to treat elevated intracranial pressure. Edema may be secondary to the disease process, surgical process, necrosis and inflammation, viral particles, metastatic tumor cells. Symptoms may include, but are not limited to, severe headache, confusion, lethargy, unresponsiveness, or focal neurological deficits. Participants generally take prophylactic high-dose steroids, but the dose may need to be increased. Edema that does not respond to aggressive treatment can lead to permanent nerve damage.
- Infection
  Any surgical procedure carries a risk of infection. This risk may be increased by injecting the virus.
- Other risks

8. Adverse event (AE)

8.1. Document of adverse events

All AEs will be recorded in a Case Report Form (CRF), either during clinical follow-up or voluntarily reported by the patient and their families, and graded according to NCI-CTCAE v5.0. If the investigator determines that a dose-related SAE (grade 3-4) has occurred, the safety committee will evaluate the AE and make changes to the admission of subsequent patients or even stop the recruitment of subsequent participants. AE is recorded until 12 weeks after viral injection as required by the protocol.

8.2. Time to collection of adverse events

Collection of all AEs will begin on the day of virus injection. If investigators believe an event is relevant to this study, the event should be collected at any time after using the study product.

8.3. Definition of adverse events

Refer to the occurrence or worsening of any adverse medical event (e.g., signs, symptoms, disease, syndrome, intercurrent illness, abnormal laboratory findings) relative to baseline levels following the administration of an intervention (pharmacotherapy). AE is not necessarily causally related to drug use. Therefore, AE can be any adverse and/or unexpected sign (including abnormal laboratory results), symptoms, and diseases that are not associated with drug use. Common events/symptoms during surgery (e.g., pain, headache, changes in standard blood pressure, constipation, etc.) are usually within the normal range. Long-term, latent, disease-related conditions that have not changed from baseline are not considered AEs. Exacerbations of underlying chronic conditions will be assessed for their "severity" and, if determined to be "severe," will be reported as SAE at the appropriate location on the form. SAE includes the following:

- Fatal
- Life-threatening

- Request or extend hospital stay
- Disability
- Can cause congenital anomalies/birth defects

In addition, any significant medical event can and should be reported to the sponsor as a SAE if deemed necessary by the principal investigator.

8.4. Grade of adverse events and relevance of study drugs.

- Level 1: Unrelated
- Level 2: Unlikely
- Level 3: Possible
- Level 4: Likely
- Level 5: Certain

The association between adverse events and Ad-TD-nsIL12 will be determined by the principal investigator, and any level 4 or 5 association will be considered relevant to the treatment of Ad-TD-nsIL12.

8.5. Unexpected adverse events

Any drug will be inconsistent with the specificity or severity of the drug described in the current investigator's manual.

8.6. Report of serious adverse events

All accidental, life-threatening or fatal incidents related to the research product must be reported in writing to the project sponsor within 24 hours of the event (the next business day). All SAEs defined in the protocol that are considered drug-related must submit a written report to the program sponsor within 5 business days of occurrence. Serious adverse effects must be followed until clinical recovery is complete, laboratory testing returns to baseline, the progression of the event has stabilized, or the event has been acceptably resolved.

9. Ethics

9.1. Ethical considerations

- The protocol and all the required documents attached will be submitted to the Ethics Committee of Sanbo Brain Hospital, Capital Medical University for approval before starting the study. Any modifications to the protocol will be submitted to the ethics committee for approval.
- All patients in this study will be given a consent form describing this study and will be provided with sufficient information to allow participants to make a decision about their participation in this study. This consent will be submitted with the protocol for review and approval by the ethics committee.

9.2. Information provided to patients and informed consent

- All participants in this study will be given a consent form describing this study and provided with sufficient information to make a decision about their participation in this study. Before the patient join any study procedure, formal consent must be obtained from the patient or a legally acceptable agent using an approved consent form. The procedure will be carried out in accordance with ICH specifications. The investigator will retain the informed consent form in the investigator's file.
- Patients under the age of 12 do not have to sign an informed consent form, and they will listen to information about the treatment protocol in order to participate in decision-making and understand treatment options. The patient's parents will sign an informed consent form. Patients over the age of 12 can sign an informed consent form with parents' signature.
- Patients and parents will be informed that participation is voluntary and can

withdraw from the study at any time without any impact on future medical care.

- The informed consent form shall not modify the information content without the written approval of the ethics committee and the sponsor.
- The informed consent form includes information about the need to review medical records and informs the patient that stored laboratory samples (e.g., blood, tissue, etc.) will be used for analysis in the future, and that additional blood tests and preserved tumor tissue samples will be used for genotyping and tissue bank storage as appropriate.

9.3. Patient confidentiality

- In order to respect the privacy of the patient, in the form of the patient information record, the patient will be identified using the assigned patient number.
- The investigator will allow the monitor or auditor or sponsor-designated collaborator to access the patient's original medical records for verification of the data in the CRF. Patient confidentiality will be carried out in accordance with appropriate laws.

9.4. Modification and violation of the test protocol

- The investigator will conduct the test according to the trial protocol after approval by the competent authority and ethics committee.
- The agreement will not be modified without the approval of the ethics committee and sponsor. All modifications to the protocol require prior approval by the ethics committee, except in cases where changes must be made to avoid immediate risk to the patient.
- Any violations of the scheme will be recorded in the CRF and the original records.

9.5. Early termination of the trial

The study may be terminated early if the manufacturer, investigators or regulator of Ad-TD-nsIL12 deem it justified. If the trial is terminated prematurely, the sponsor should report the reason for the termination or suspension. Reasons for early termination or suspension of the trial include, but are not limited to:

- Patients present with serious risks not foreseen by the protocol.
- Unable to recruit a sufficient number of patients.
- Insufficient compliance with the protocol.
- Significant changes or pauses in drug development programs.

10. Statistical methods

10.1. Study endpoints

- Primary endpoint
  The maximum tolerated dose (MTD) of Ad-TD-nsIL12. (According to the criteria of NCI-CTCAE V5.0, MTD will be defined as doses below the dose that causes DLT. If no DLT has occurred at D3, the trial is terminated and MTD is not defined.)
- Secondary endpoints
  Tumor response based on tumor size change on continuous MRI scans after injection, time to progression, viral activity based on sample analysis, and immunogenicity based on adenovirus antibodies in serum. Overall survival of the patient. Due to the small number of participants, it is expected that the secondary endpoint will only be used to provide reference trends for future phase II efficacy studies.

10.2. Estimation of sample size

Sample size was determined by the incidence of DLT in three dosing cohorts, each with 3 to 6 assessable participants. If DLT is not observed, at least nine participants will be included if MTD is not reached. Admission to nine participants was expected to take 10

to 12 months; 18 patients about 15-20 months.

10.3. Safety

Patients who do not complete the study, for any reason, will include all available data prior to termination of the study in the safety analysis. AEs will be summarized by population and dose group, and tabulated by severity, relationship with Ad-TD-nsIL12, and causation. In addition, SAEs, AEs that may be related to Ad-TD-nsIL12, and AEs that are not related to Ad-TD-nsIL12 will be aggregated separately. Concurrent conditions will be listed and may be identified as possible confounding factors in the treatment-response relationship. Follow-up and treatment for AE will also be documented. Summary data includes univariate statistics for mean, standard error, percentage, and median. AE-related clinical laboratory results will provide descriptive statistics on selected laboratory parameters based on population, patients, study dates, and dose groups.

10.4. Effectiveness

In this study, tumor response will be assessed according to RAPNO criteria. Response to treatment will be determined by the size of the brain tumor from baseline, at 28 days, 8 weeks, and 12 weeks after the end of virus administration (if the patient still has unplanned radiological follow-up, the observation of tumor response period continues). Changes in clinical disease status and steroid use will be considered when assessing changes in tumor size. The size of the tumor in the same patient will be calculated by MRI scan. The percentage of participants whose symptoms improved or stabilized after the end of the injection was also pooled using a 95% confidence interval. Time to disease progression, KPS or LPS score, and survival can also be assessed using Kaplan-Meier analysis.

11. Record-keeping

After the start of the study, investigators must keep dosing records, participant exclusion logs, signed informed consent forms, electronic CRFs, all correspondence, and relevant important documents for more than 2 years.