
AAV gene therapy for autosomal recessive deafness 9: a single-arm trial

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6. Protocol Amendments and Reasons (Pages 77-78).

Sequences of Anc80L65-OTOF

Anc80L65-*OTOF* consisted of Anc80L65-*OTOF*-N, the 5' terminal segment of the human *OTOF* coding sequence, and Anc80L65-*OTOF*-C, the 3' terminal segment of the human *OTOF* coding sequence.

>Anc80L65-*OTOF*-N sequence

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CCTGCAGGCAGCTGCGCGCTCGCTCGCTCACTGAGGCCGCCCGGGCGTCGGGGCGACCTT
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GCGCAGCTGCCTGCAGG

>Anc80L65-OTOF-C sequence

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CCTCAGTGAGCGAGCGAGCGCGCAGCTGCCTGCAGG

Supplementary Videos

All videos were authorized by participants or their guardians with assigned informed consents. To protect the privacy, participant faces are coded and names were hidden.

Supplementary Video 1: Participant 4 was able to have daily communication with her mother at 4 months after treatment.

Without the cochlear implant and lipreading, participant 4 was able to communicate with her mother 4 months after treatment.

Supplementary Video 2: Participant 8 heard his father's voice and recognized the words at 1 month after treatment.

Without the cochlear implant and lipreading, participant 8 could distinguish such words as "scissors", "excavator", "tomato" and "banana" said by his father with the ear treated by AAV-*OTOF* after 1 month.

Supplementary Video 3: Adult participant 9 heard and identified the sound at 1 month after treatment.

Without the cochlear implant and lipreading, participant 9 could distinguish between handclapping sounds and language, and was able to correctly identify the number of spoken words or handclapping after 1 month.

List of Investigators

The principal investigators from the 5 centers were listed below.

Center name	Investigator	Role
Shandong Second Provincial General Hospital	Prof. Lei Xu	Leading Site
Union Hospital Tongji Medical College, Huazhong University of Science and Technology	Prof. Yu Sun	Participating site
Nanjing Drum Tower Hospital, Affiliated Hospital of Medical School, Nanjing University	Prof. Xia Gao	Participating site
Xijing Hospital, The Fourth Military Medical University	Prof. Dingjun Zha	Participating site
Ningbo No.2 Hospital	Prof. Kai Wang	Participating site

Primers

Primers used in the Minigene assay.

Primer name	Sequences (5'-3')
78662- <i>OTOF</i> -F	GGAGAGGAGAGAGTGGGAAC
78875- <i>OTOF</i> -F	GGTGGGGAATGCACTCTAGG
81173- <i>OTOF</i> -R	TCCAGTTGCGTCCTCACCAG
81383- <i>OTOF</i> -R	GCAGCTTCTGCAGGAAGTTC
<i>OTOF</i> -mut-F	GCCAGGGGCCAGCAGAGGTGGGGTCCTGGGG
<i>OTOF</i> -mut-R	CCCCAGGACCCACCTCTGCTGGCCCCCTGGC
pcMINI- <i>OTOF</i> -KpnI-F	GGTAGGTACCCCCATCCTCCTGTGGCCTGG
pcMINI- <i>OTOF</i> -XhoI-R	TTTCCTCGAGGAGATGAAGTTTTGCTCGGT
pcMINI-F	ACTTAAGCTTATGAGTGGGCTTTGGGGTGGCCGGTT
pcMINI-R	TAGAAGGCACAGTCGAGG

Study Protocol

An Investigator-Initiated Trial to Evaluate the Safety, Tolerability, and Effectiveness of OTOV101N + OTOV101C Injection in the Treatment of Patients with OTOF Gene Mutation-Related Deafness

Study Drug : OTOV101N + OTOV101C Injection

Protocol No. : OTOV101-IIT-101

Leading Site of the Clinical Study : Shandong Second Provincial General Hospital

Principal Investigator : Director Xu Lei

Cooperative Institution : Suzhou Otovia Therapeutics Co., Ltd.

Version No. : V1.5

Version Date: : June 17, 2024

Confidentiality Statement

This clinical study protocol is a confidential document and is provided for the purpose of this clinical study only. It should not be disclosed to anyone other than the participating investigators and members of the institutional review board.

Statement and Signature Page
Clinical Study Institution

I will conduct this study and perform the investigator responsibilities in strict accordance with the Declaration of Helsinki, the principles of GCP, the principles of ICH-GCP, and the established clinical study protocol; reasonably design the study protocol and submit it to the Ethics Committee as required; make scientific medical judgments about the study process and strive to protect the rights and privacy of subjects. record study data in a timely, comprehensive and careful way and timely report adverse events (AEs) and serious adverse events (SAEs). I'm aware of the requirements for correctly reporting SAEs and I will record and report them as required. I hereby warrant that the subjects will receive timely and appropriate treatment in case of any AE during the study, and that appropriate treatment measures will be taken immediately for the subjects and corresponding reporting will be made in case of any SAE.

Leading site of the clinical study: Shandong Second Provincial General Hospital

Principal investigator (Printed name): Xu Lei

Signature: _____

_____DD/MM/YYYY

Statement and Signature Page**Clinical Study Institution**

I will conduct this study and perform the investigator responsibilities in strict accordance with the Declaration of Helsinki, the principles of GCP, the principles of ICH-GCP, and the established clinical study protocol; reasonably design the study protocol and submit it to the Ethics Committee as required; make scientific medical judgments about the study process and strive to protect the rights and privacy of subjects. record study data in a timely, comprehensive and careful way and timely report adverse events (AEs) and serious adverse events (SAEs). I'm aware of the requirements for correctly reporting SAEs and I will record and report them as required. I hereby warrant that the subjects will receive timely and appropriate treatment in case of any AE during the study, and that appropriate treatment measures will be taken immediately for the subjects and corresponding reporting will be made in case of any SAE.

Clinical study institution: _____

Signature of the principal investigator/Date: _____ DD/MM/YYYY

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Abbreviation

English abbreviation	English term
AAV	Adeno-Associated Virus
ABR	Auditory Brainstem Response
AE	Adverse Event
AI	Aromatase Inhibitor
ASSR	Auditory Steady-State Response
CI	Cochlear Implantation
CM	Cochlear Microphonic
CT	Computed Tomography
DFNA	Autosomal Dominant Non-syndromic Hearing Loss
DFNB	Autosomal Recessive Non-syndromic Hearing Loss
DFNB9	OTOF-related Deafness
DFNX	Mitochondrial Inheritance and X-linked Inheritance
DPOAE	Distortion Product Otoacoustic Emissions
eCRF	Electronic Case Report Form
FAS	Full Analysis Set
HBV	Hepatitis B Virus
HBsAg	Hepatitis B Surface Antigen
HCV	Hepatitis C Virus
HIV	Human Immunodeficiency Virus
ICF	Informed Consent Form
IHC	Inner Hair Cell
IIT	Investigator-Initiated Trial
MRI	Magnetic Resonance Imaging
NA	Not Applicable
NMPA	National Medical Products Administration
OHC	Outer Hair Cell
OMIM 603681	OTOF Gene
PT	Preferred Term
QC	Quality Control
SAE	Serious Adverse Event
SS	Safety Analysis Set
SUSAR	Suspected Unexpected Serious Adverse Reaction

Abstract

Protocol name	An Investigator-Initiated Trial to Evaluate the Safety, Tolerability, and Effectiveness of OTOV101N + OTOV101C Injection in the Treatment of Patients with OTOF Gene Mutation-Related Deafness
Version No./Version date:	1.5/June 17, 2024
Study phase	Investigator-initiated trial (IIT)
Name of study drug	OTOV101N + OTOV101C Injection
Leading site of the clinical study	Shandong Second Provincial General Hospital
Principal investigator	Director Xu Lei
Cooperative institution	Suzhou Otovia Therapeutics Co., Ltd.
Study drug	OTOV101N + OTOV101C Injection Titer of study drug: 2.8×10^{13} vg/mL; Route of administration: Intracochlear injection via round window membrane; Dosage regimen: Each subject will receive the intracochlear injection of adeno-associated virus (AAV) at the dose of 4.2×10^{11} vg/ear/virus to 5.6×10^{11} vg/ear/virus (the titer of OTOV101N + OTOV101C Injection is 2.8×10^{13} vg/mL, with each injection volume of 15~20 μL/ear, and after 1: 1 mixing, intracochlear injection is performed, with the total volume of 30~40 μL/ear). For subjects with no cochlear implants on either side, unilateral intracochlear injection or bilateral intracochlear injection may be performed as assessed by the investigator. For intracochlear injection, the investigators will decide if the second intracochlear injection should be conducted based on dose of the first injection by considering anatomical structure of artificial cochlea and drug loss. The timing of the second

	injection can be determined depending on the surgical recovery from the first injection. • Shelf life: 2 years; • Transport and storage conditions: Not higher than -60°C.
Target population	Patients aged 1 year and older with severe or greater deafness due to OTOF gene mutations who have remaining inner hair cells.
Study duration	The screening period will be no longer than 60 days and the clinical study will end after the last subject has been treated with OTOV101N + OTOV101C Injection and completed the 5-year follow-up.
Study objectives	1) To evaluate the safety and tolerability of OTOV101N + OTOV101C Injection in patients with OTOF gene mutation-related deafness. 2) To evaluate the effectiveness of OTOV101N + OTOV101C Injection in patients with OTOF gene mutation-related deafness.
Overall design	Multicenter, open-label, non-randomized controlled interventional clinical study
Study design	General principle This is an investigator-initiated trial to evaluate the safety, tolerability, and effectiveness of OTOV101N + OTOV101C Injection in the treatment of patients with OTOF gene mutation-related deafness. The purpose of the study is to evaluate the safety, tolerability, and effectiveness of OTOV101N + OTOV101C injection in the treatment of patients with OTOF gene mutation-related deafness. The successfully enrolled subjects will be started on glucocorticoids on D-3, and can be admitted to the hospital on D-3~D-1 in preparation for inner ear gene therapy. On the day of surgery (D0), subjects will receive the intracochlear injection of drugs for gene therapy via the round window after strict sterilization of the surgical area and general anesthesia. Each subject will receive the intracochlear injection of adeno-associated virus (AAV) at the dose of 4.2×10^{11} vg/ear/virus to 5.6×10^{11} vg/ear/virus (the titer of OTOV101N+OTOV101C

	Injection is 2.8×10^{13} vg/mL, with each injection volume of 15~20 μL/ear, mixed at a 1:1 ratio for intracochlear injection, with the total volume of 30~40 μL/ear). For subjects with no cochlear implants on either side, unilateral intracochlear injection or bilateral intracochlear injection can be conducted as assessed by the investigator. For intracochlear injection, the investigators will decide if the second intracochlear injection should be conducted based on dose of the first injection by considering anatomical structure of artificial cochlea and drug loss. The timing of the second injection can be determined depending on the surgical recovery from the first injection. After receiving intracochlear injection, subjects need to stay in the hospital for observation for at least 3 days or the routine length of stay for diagnosis and treatment determined by the study site, and are allowed to return home after recovery from surgery, followed by a 5-year follow-up. The clinical study will not be concluded until the last subject is treated with OTOV101N + OTOV101C Injection and completes the 5-year follow-up. All subjects will return to the hospital (except encountering force majeure events) during the study process for safety and efficacy assessments at time points specified in the protocol (W1 $\pm$ 1d, W2 $\pm$ 3d, W3 $\pm$ 3d, M1 $\pm$ 3d, M2 $\pm$ 3d, M4 $\pm$ 6d, M6 $\pm$ 6d, M9 $\pm$ 6d, M12 $\pm$ 14d, M18 $\pm$ 14d, M24 $\pm$ 14d, M30 $\pm$ 14d, M36 $\pm$ 14d, M42 $\pm$ 14d, M48 $\pm$ 14d, M54 $\pm$ 14d, M60 $\pm$ 14d/EOS/Unscheduled). <i>Selection of study ear:</i> <i>Unilateral intracochlear injection: The ear with more severe hearing loss will be selected as the study ear. If the hearing condition is the same in both ears, the left ear will be selected as the study ear. If one side has already received a cochlear implant, the other side is selected as the study ear. Bilateral intracochlear injection: For subjects with no cochlear implant on either side, bilateral intracochlear injection may be performed.</i>
Sample size	Planned sample size is 25 subjects.

Inclusion criteria	<u>Subjects must meet all of the following inclusion criteria to be enrolled in the study:</u> 1) Age ≥ 1 year at the time of signing the ICF; male or female; 2) Diagnosis of OTOF related deafness is established as per the following criteria: a) Hearing loss confirmed by auditory testing and auditory brainstem response (ABR) test (test reports within 1 month before the signing of ICF are acceptable); b) Presence of homozygous or compound heterozygous mutations in the OTOF gene confirmed by genetic testing; 3) Auditory testing: Bilateral severe ($65 \text{ dB} \leq \text{hearing threshold} < 80 \text{ dB}$) or profound ($80 \text{ dB} \leq \text{hearing threshold} < 95 \text{ dB}$) or complete (hearing threshold $\geq 95 \text{ dB}$) hearing loss; (if ABR test results are “not produced”, subjects with bilateral hearing threshold $< 65 \text{ dB}$ may be enrolled as judged by the investigator); 4) Test results of vital signs, physical examination, laboratory tests (blood routine examination, blood biochemistry and urine routine examination, coagulation function, etc.), 12-lead ECGs and other tests are all normal, or the abnormality is judged by the investigators as having no clinical significance; 5) Subjects and their guardians sign the ICF.
Exclusion criteria	<u>Subjects meeting any of the following criteria should not be enrolled:</u> 1) Patients who have previously experienced serious allergic reactions to any drug or its components in this study ($\geq$ Grade 3 Severe AE as per NCI-CTCAE v 5.0); 2) Any prior gene therapy or blood neutralizing antibody level greater than 1:128; 3) Patients with systemic diseases or having received relevant treatment that may affect hearing or surgical operation;

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| | <ul style="list-style-type: none">4) Patients intolerable to general anesthesia;5) Patients with inner ear malformation;6) Bilateral cochlear implantation, or history of major inner ear surgery (as judged by the investigator) (except unilateral cochlear implantation);7) Patients with other mutations in deafness gene that affect judgment of OTOF gene therapy effect;8) Patients with Meniere's disease;9) Patients who are routinely treated with ototoxic drugs for other diseases;10) Patients with congenital deafness due to the presence of other non-genetic factors associated with hearing loss at birth;11) Subjects who are receiving or are likely to receive immunosuppressive therapy other than the study drug of this study;12) Subjects with hypersensitivity or intolerance to glucocorticoids;13) History of malignancy or meningitis;14) Subjects with persistent or active infection, positive HBsAg results and HBV DNA titer in peripheral blood higher than the lower limit of detection, positive HCV antibody test results and HCV RNA titer in peripheral blood higher than the lower limit of detection, positive HIV antibody test results or other immunodeficiency diseases, and positive syphilis test results;15) Subjects of childbearing potential who refuse to use effective contraception (hormonal or barrier contraception or abstinence) from signing the informed consent to 12 months after injection of AAV;16) Female subjects of childbearing potential with positive blood pregnancy test results or who are pregnant or lactating;17) Subjects who have participated in any other clinical trial within 4 weeks prior to the first dose and have received administration or treatment (including drug and device clinical trials, excluding non-interventional studies);18) Subjects who are unwilling or unable to comply with this protocol; |
|--|--|

	19) Subjects who, in the opinion of the investigators, are unable to participate in the study or to complete the subsequent study due to any medical conditions.
Withdrawal criteria	1) Subjects wrongly included, judged by the investigator as not meeting inclusion criteria or meeting exclusion criteria; 2) Subjects failing to receive gene therapy after enrollment; 3) Occurrence of an AE, which is considered by the investigators, cooperative institution or medical monitor as having caused unacceptable consequences or risks to the subjects; 4) The safety of the subject, as considered by the investigators, is compromised at any time during the trial; 5) Subjects with poor compliance who, as assessed by the investigators, are not suitable to continue this study; 6) Other reasons that the investigators consider it inappropriate for the subject to continue the trial; 7) Withdrawal of informed consent at any time by the subject or his/her guardian (if any); 8) Subjects lost to follow-up. When a subject withdraws prematurely from the study, the investigator should ask the subject whether he/she is willing to continue follow-up visits and whether he/she agrees to record the discussion results in the medical history and eCRF. Where a subject is lost to follow-up, the investigator should make every effort to reach out to the subject or his/her relative, get acquainted with the reason for his/her withdrawal from the study, complete the last assessment, and record the reason for the subject's withdrawal from the study. Where a subject withdraws from the study due to an AE, the AE should be followed up until the subject returns to normal or the baseline level, or the status of the subject is stable or the subject is lost to follow-up, and corresponding records should be made.

Termination criteria	1) Under the premise of fully protecting the rights and interests and safety of subjects, the investigators or cooperative institution demands termination; 2) Regulatory authorities or the Ethics Committee order termination of the study due to reasons such as significant safety risks of the study drug; 3) Other reasons that the cooperative institution or the investigator considers inappropriate to continue the study.
Evaluation indicators	➤ Safety evaluation indicators: All AEs throughout the study are assessed and graded according to NCI-CTCAE v 5.0. Safety evaluation indicators include: 1) Incidence and severity of AEs and their correlation with the study drug; 2) Physical examination, including cranial nerve testing; 3) Laboratory test indicators (blood routine examination, urine routine examination, blood biochemistry, coagulation function, etc.), vital signs and ECG examination; 4) Neutralizing antibodies detection and AAV testing in peripheral blood. ➤ Efficacy evaluation indicators: According to the degree of hearing restoration of subjects, the investigator should test the hearing of subjects according to the required follow-up time points, and the content of the test mainly include: 1) Hearing assessment: Behavioral audiometry, ABR, ASSR, distortion product otoacoustic emission (DPOAE) response; 2) Cochlear microphonic (CM) potentials; 3) Tympanogram examination.
Concomitant medication	➤ Concomitant medication 1) Prednisone or equivalent oral corticosteroids are administered for the first 3 days of receiving gene therapy until W8. The maximum prednisone dose is no more than 40 mg/day. 2) To prevent infection, prophylactic antibiotics should be administered until postoperative day 3, and the specific dosage regimen and duration of

	administration should be based on the study institution's practice. ➤ Medications and therapies used concomitantly during the study as appropriate 1) Chronic medications required prior to enrollment for concomitant diseases that, in the judgment of the investigators, will not pose a risk of ototoxicity; 2) Symptomatic treatment for drug toxicities. ➤ Prohibited medications during the study 1) During the study, concomitant medications other than those specified in the protocol and those that may be used concomitantly as appropriate, are prohibited. When a subject needs to use other drugs during the study, he/she should consult the responsible investigator beforehand, and inform the responsible investigator truthfully afterwards even if he/she fails to reach the investigator due to special reasons.
Statistical analysis	Statistical methods: ➤ Statistical analysis software In this study, statistical analyses will be performed using SAS Version 9.4 or newer versions. ➤ Analysis set Full Analysis Set (FAS): All subjects who have received the study drug; the FAS is mainly used for the analysis of demographic and baseline data, subject distribution and protocol violations. Safety Analysis Set (SS): All subjects who have received the study drug and have at least one post-treatment safety evaluation data; SS is primarily used for the analysis of safety data. Effectiveness Analysis Set (EAS): All subjects who have received the study drug and completed at least one post-baseline hearing function test. EAS is primarily used for the analysis of efficacy data. ➤ Overall analysis plan

	Baseline data will be summarized in the Safety Analysis Set. For continuous variables, the statistical description of mean, standard deviation, quartiles, maximum and minimum values will be performed. For categorical variables, frequency and corresponding percentage will be described. Safety analysis will be performed in the Safety Analysis Set and summarized by clinical visit. Safety analyses include otologic examinations, laboratory tests, and other reported otologic or systemic AEs. AEs will be summarized by type and frequency. Efficacy analyses will be performed in the EAS, where quantitative efficacy indicators will be summarized by clinical visit for the treated ear and contralateral ear, and trends in change from baseline will be calculated, including means, standard deviations, and 95% confidence intervals for changes. Qualitative efficacy indicators will be summarized by clinical visit for the treated ear and contralateral ear, and changes from baseline will be described using a cross-tabulation table before and after treatment. This study is an exploratory study and no formal hypothesis testing will be performed.
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Study Flow Chart

Test item	Screening period	D-3~D-1 [@]	D0 ^{^^}	D1	D3	W1 [*]	W2	W3	M1 [*]	M2	M4	M6	M9	M12 ± 6d/EOS [※] /Unscheduled	M18 ± 14d/ every 6 visits until M60 ± 14d
	60 days		Day of surgery		/	±1d	±3d	±3d	±3d	±3d	±6d	±6d	±6d		
Signed ICF	x														
Demographic data ¹	x														
Medical history ²	x														
Inclusion/Exclusion criteria ³	x														
Vital signs ⁴	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x
Physical examination ⁵	x	x		x	x	x	x	x	x	x	x	x	x	x	x
Weight ⁶	x	x					x		x						
ECG ⁷	x	x			x									x	x
Blood pregnancy test ⁸	x	x												x	
Virology screening test ⁹	x														
Blood routine examination and urine routine examination	x	x			x	x	x	x	x	x	x	x	x	x	x
Blood biochemistry	x	x			x	x	x	x	x	x	x	x	x	x	x
Coagulation function test	x	x			x									x	
Brain MRI/CT ¹⁰	x														

Test item	Screening period	D-3~D-1@	D0▲▲	D1	D3	W1*	W2	W3	M1*	M2	M4	M6	M9	M12 ± 6d/EOS*/ Unscheduled	M18 ± 14d/ every 6 visits until M60 ± 14d
	60 days		Day of surgery		/	±1d	±3d	±3d	±3d	±3d	±6d	±6d	±6d		
Auditory testing (behavioral audiometry, tympanogram, DPOAE, CM, ABR, ASSR examination) ^{11▲▲}	x						x		x	x	x	x	x	x	x
Venous blood draw (neutralizing antibody detection) ¹²	x				x	x	x	x	x	x	x	x	x	x	X ¹²
Venous blood draw (AAV testing) ¹²				x	x	x	x	x	x	x	x	x	x	x	
Prophylactic antibiotic administration ¹³			x	x	x										
Glucocorticoid dispensing		x	x		x	x	x	x	x						
Oral glucocorticoid administration ¹⁴		x	x	x	x	x	x	x	x	x					
Intracochlear injection			x												
Genetic testing ¹⁵	x														
AEs ¹⁶			x	x	x	x	x	x	x	x	x	x	x	x	x
Concomitant medication	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x

Note:

*: W1: refers to the 7th day after surgery, and so on in a similar fashion; M refers to the month, which is calculated as 28 days per month;

@: Subjects may go to the hospital to be hospitalized on D-3~D-1, and the specific time may be reasonably arranged by the investigators upon evaluation based on the actual situation of the subjects; the examination required in this clinical visit is the hospitalization examination on hospitalized subjects;

&: This study allows retesting of all test items during the screening period. For retesting of abnormal clinically significant examinations, the investigators should discuss with the study team to determine whether to adopt the retest results as the basis for judging the subjects meeting the inclusion and exclusion criteria;

▲▲: Second injection operation: The investigators will decide if the second intracochlear injection should be conducted based on dose of the first injection by considering the anatomical structure of the artificial cochlea and drug loss. The timing of the second injection will be decided by recovery status of the first injection. Auditory testing is required before the second injection (the test results within 1 month before surgery is acceptable). The second injection is performed on D-3~D-1 and D0 visits according to the flow chart. The follow-up visit (D1~M60 ± 14d/EOS) is performed according to the time of the second surgery.

#: Subjects will be allowed to undergo 1 rescreening for this study and each screening will be assigned a new screening number. Subjects meeting all the inclusion criteria and falling under none of the exclusion criteria can participate in this study.

※: EOS visit: Occurred between D1-M12 visits, hearing tests are performed as per requirements of M12 visit; occurred between M18-M60 visits, hearing tests are performed as per requirements of M18-M60 visits.

1. Demographic data: Including gender, age (date of birth) and ethnicity of subjects;
2. Collection of date on medical history: Past medical history and treatment history, inner ear surgery history, allergy history, and tumor history should be recorded.
3. Review of inclusion/exclusion criteria.
4. Vital signs: Vital signs, including body temperature, blood pressure, pulse, and respiratory rate, are measured after subjects have rested quietly for at least 5 min. The same position should be maintained at each visit as far as possible.
5. Physical examination: Including general condition, skin mucosa, lymph nodes, head, neck, chest, abdomen, spine, limbs and neurological examination. In addition to general examination, neurological examination focuses on trigeminal nerve, facial nerve, vagus nerve, glossopharyngeal nerve, and accessory nerve functions as well as taste function.
6. Weight: Body weight should be measured during the screening period, on D-3~D-1, at W2, W4 (i.e., M1), W6, and if necessary (patient self-measurement results are acceptable);

7. ECG: Examinations are performed after subjects have rested quietly in a recumbent position for at least 5 min; including heart rate, PR interval, QRS interval, QT interval, and QTc interval.
8. Blood pregnancy test: This test is only performed for female subjects of childbearing age who are capable of childbearing; it may be performed at any time if required.
9. Virology screening tests include: HBsAg test, HCV test, HIV antibody test, and syphilis antibody test. For HBsAg-positive subjects, HBV-DNA testing is required. HCV antibody-positive subjects should receive HCV-RNA test. Test results from our hospital or other hospitals within 60 days before administration are acceptable;
10. Brain MRI/CT: Brain MRI/CT should be performed when clinically indicated during the screening period and subsequent follow-up visits and judged by the investigators as necessary; in the screening period, for patients with cochlear implants, brain MRI is not required if there are previous brain MRI results (no time window is required) (whether it is necessary to increase brain CT is based on the investigator's judgment); brain CT is required if there are no previous cranial MRI results; and cranial MRI is required for patients without cochlear implants. During subsequent follow-up visits, whether brain MRI or brain CT examination should be performed is at the investigator's discretion based on the subject's actual condition.
11. Auditory testing (behavioral audiometry, tympanogram, DPOAE, CM, ABR, ASSR examination): Among the visits during screening period and in postoperative W2, M1, M2, M4, M6, M9, M12/EOS visits, the auditory testing during the screening period is mandatory, and at subsequent visits, it may be decided whether to increase/decrease the auditory testing visits and increase/decrease the auditory test items based on the subjects' willingness of cooperation and the assessment of investigators. Auditory testing is required before the second injection (test results within 1 month before surgery are acceptable). Hearing test at M18-M60 visit includes: Behavioral audiometry/pure tone audiometry and ABR test; EOS visit occurred between M18-M60 visits; hearing tests are performed as per requirements of M18-M60 visits
12. Neutralizing antibody detection: Venous blood will be drawn for neutralizing antibody detection in the visits during screening period, on the postoperative Day 3, in W1, W2, W3, M1, M2, M4, M6, M9, M18, M24, M36, M48 and M60//EOS visits (**neutralizing antibody detection is not required on postoperative Day 1**) until the test result of neutralizing antibody turns out to be negative . AAV testing: Venous blood will be drawn for AAV testing on postoperative Day 1, Day 3, in W1, W2, W3, M1, M2, M4, M6, M9, and M12/EOS visits (**AAV testing is not required before the day of surgery**).
13. Prophylactic antibiotic administration: To prevent infection, prophylactic antibiotics should be administered until postoperative day 3, and the specific dosage regimen and duration of administration should be based on the study institution's practice.

14. Oral glucocorticoid administration: Subjects receive oral prednisone 0.5 mg/kg body weight/day or equivalent of other corticosteroids starting 3 days prior to gene therapy. The dose is increased to 1 mg/kg body weight/day from the day of surgery to W1, decreased to 0.5 mg/kg body weight/day at W2~W3, decreased to 0.25 mg/kg body weight/day at W4~W5 and decreased to 0.125 mg/kg body weight/day at W6~W8. Then, the drug is discontinued. Maximum prednisone dose is no more than 40 mg/day. The actual dose administered is assessed by the investigator based on subjects' actual condition.
15. Genetic testing: If a subject is unable to provide the genetic test report for deafness or the genetic test report for deafness does not contain parental verification, venous blood should be collected from the subject for molecular diagnosis to confirm the type of gene defect, and venous blood will be collected from the parents of the subject for pedigree verification.
16. Monitoring is performed throughout the trial in such forms as safety assessments, observations, and subject (self-)reports.

1. Study Background

1.1. R&D background

1.1.1. Hereditary deafness and its effects

Hearing loss is the fourth leading disabling factor worldwide^[1], causing communication disorders, social isolation, employment discrimination, lower degree of education and lower income, seriously affecting the quality of life, and increasing the incidence of senile dementia and depression with the increase of patients' age^[2, 3]. According to the WHO's world health statistics report 2021, there are a total of 466 million people worldwide suffering from disabling hearing loss (auditory threshold > 35 dB HL), accounting for approximately 5% of the world's population. In China, according to the main data bulletin of the Second China National Sample Survey on Disability in 2006, there are 20.04 million people with hearing disabilities, accounting for 24.16% of the total number of disabled people, ranking second among the causes of disability^[4]. The main causes of hearing loss are environmental and occupational noise exposure, the use of ototoxic drugs, genetic mutations and defects, and senile degeneration^[3].

The incidence of neonatal deafness in China is 1/1,000 – 3.47/1,000, and genetic factors account for 50–60%^[5,6]. Hereditary deafness is a major cause of prelingual hearing loss^[7]. Hereditary hearing loss is divided into two phenotypes: Syndromic and non-syndromic hearing loss; the former is accompanied by lesions in other organ systems in addition to hearing disorder, accounting for 20% of hereditary hearing loss, such as Usher syndrome, which encompasses visual system lesions in addition to hearing disorder; the latter accounts for 80% of hereditary deafness, and the pathological phenotype only involves with the auditory system, which is the main phenotype of hereditary hearing loss. According to the pattern of inheritance of genetic lesions, non-syndromic deafness is subdivided into autosomal dominant deafness (DFNA), autosomal recessive deafness (DFNB), mitochondrial inheritance and X-linked inheritance (DFNX), of which DFNB accounts for 80% of the main genetic phenotypes of non-syndromic hearing loss^[7], involving more than 120 genes and more than 6,000 pathogenic variants^[8].

1.1.2. OTOF-related deafness (DFNB9)

Mutations in the OTOF gene (OMIM 603681) are the main cause of DFNB9 (OMIM 601071) hearing loss. OTOF, located on chromosome 2p23.3, is a protein with six calcium-binding C2 domains, belonging to the FER-1 protein family^[9]. OTOF has five main transcripts and encodes five OTOF

protein isoforms, respectively ^[10]. Among them, isoforms 1 and 3 are expressed in brain tissue, and isoform 2 is expressed in embryonic tissue, brain tissue, heart, placenta, skeletal muscle, and kidney ^[11]. Isoform 5 is specifically expressed in human and mouse cochlear tissue ^[12, 13], and transcript 5 bypasses exon 47 and uses a stop codon in exon 48 during RNA splicing ^[13]. In mature cochlear tissue, OTOF is expressed at ribbon synapse sites between IHCs. Similar to Synaptotagmin at synapses in the CNS, OTOF interacts with calcium ions, SNAP25, Synaptaxin-1 during synaptic vesicle release to mediate the release of the excitatory neurotransmitter glutamate from the cytosol, and AMPA receptors on glutamate-binding SGNs mediate excitatory postsynaptic currents that in turn transmit auditory information ^[14-16]. OTOF gene mutations result in reduced efficiency of synaptic transmission between IHC and SGN, and loss of synchronous generation of SGN, resulting in hearing loss and speech discrimination impairment, accounting for 8% of congenital deafness ^[17]. Currently, about 220 disease-causing mutations and probable disease-causing mutations are found on OTOF ^[10], but the relationship between OTOF mutant genotype and pathological phenotype of patients with hearing loss has not been established ^[10, 18]. Homozygous disease-causing mutations and complex mutations in OTOF greatly contribute to severe and profound prelingual deafness and temperature-sensitive deafness ^[18]. The diagnostic criteria for DFNB9 and other auditory neuropathy are hearing loss, but there is distortion product otoacoustic emission (DPOAE) response. However, it has been reported that DPOAE is present in DFNB9 patients at a very young age, but disappears at 1.8 years old, suggesting that although OTOF is expressed in inner hair cells (IHC), lesions also indirectly affect outer hair cells (OHC), thus abnormal DPOAE response does not rule out DFNB9 ^[19].

1.1.3. Therapeutic strategies for DFNB9

Based on understanding the pathogenesis of OTOF gene mutations, a variety of therapies have been applied to restore hearing function, mainly including: Cochlear implant therapy and gene replacement therapy.

Cochlear implant therapy (CI): For DFNB9 patients with severe to profound deafness, cochlear implantation is an effective treatment for their hearing rehabilitation. Cochlear implants convert sound information into electrical signals through an in vitro microphone, collect information through an in vivo induction coil and transmit the collected information to cochlear implanted electrodes, which generate an electric field at the modiolus to directly stimulate the SGN. DFNB9 patients can regain language function and clearly understand utterances after receiving a cochlear implant. Cochlear

implants should be implanted as early as possible after the definite diagnosis of DFNB9 deafness, preferably no later than 2 years old for the sake of ensuring language development. In order to improve the diagnostic efficiency of DFNB9 deafness, auditory brainstem response (ABR) should be applied at early stage in combination with DPOAE and molecular diagnosis. Because DFNB9 deafness manifests as presynaptic lesions and SGN function is less affected, patients with DFNB9 deafness are under the indications for CI, which has good therapeutic effects and can restore sound perception and language recognition [20], but CI itself has many shortcomings, such as poor tone identification, difficulty in tone processing, and inability to appreciate music [21, 22]. Other treatments are needed to restore natural hearing.

Gene replacement therapy: Gene replacement therapy, also known as gene therapy, is a treatment for diseases by delivering normal genes in replacement of mutant genes. Gene therapy will play an important role in the treatment of hearing loss caused by single gene mutations. Currently, gene therapy applied for deafness is in the preclinical stage, but gene replacement therapy has been used in proof-of-concept experiments in mouse models, providing important information and paving the way for future clinical trials [23-26]. The sequence length of OTOF is 5.994 kb, which is already beyond the capacity of AAV to contain GOI (Gene of Interest) (4.7 kb). However, gene therapy and hearing restoration have been achieved in OTOF $-/-$ mice by cutting the coding sequence into two parts to package AAV separately [27, 28]. In this sense, OTOF segmentation method is also applied in this study by using dual AAV vectors for gene therapy.

1.2. Overview of study drug

Name of study drug: OTOV101N + OTOV101C Injection

Physicochemical properties:

- Titer of study drug: 2.8×10^{13} vg/ mL;
- Excipients: Tromethamine, sodium chloride, sucrose, histidine, magnesium chloride, poloxamer 188;
- Route of administration: Intracochlear injection via round window membrane;
- Injection dose: 4.2×10^{11} vg/ear/each virus to 5.6×10^{11} vg/ear/each virus through unilateral intracochlear injection;
- Transport and storage conditions: Not higher than -60°C ;
- Shelf life: 2 years.

1.3. Overview of preclinical study

1.3.1. Preclinical pharmacology studies

1) Pharmacodynamic study on research grade OTOV101N + OTOV101C Injection

In order to determine the product design of the final clinical trial, AAV vector study, OTOF cutting site study, promoter study and other studies are conducted using the Company's production system of research grade virus and data research, and the design of OTOV101N + OTOV101C Injection is completed based on these data.

- a) **AAV vector study:** Based on literature research, two foreign companies engaged in cochlear gene therapy selected AAV-No.1 and AAV-No.2 vectors for packaging their OTOF pipelines, respectively. According to the comparison of literature data (Figure 1), the infection efficiency of AAV-No.2 vector on IHCs in the cochlea of cynomolgus monkeys is significantly higher than that of AAV-No.1 vector. Therefore, AAV-No.2 vector is determined as the packaging vector for this project.

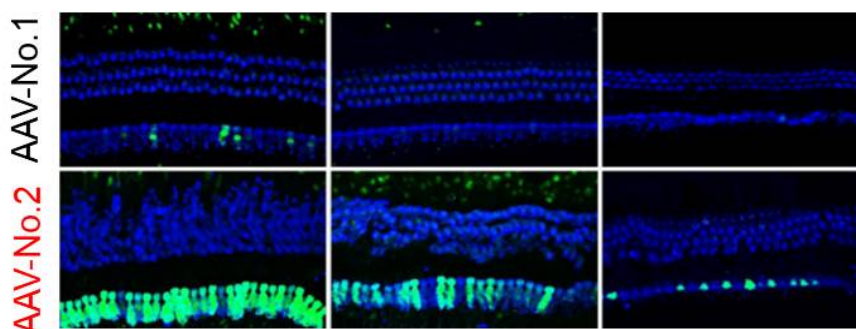


Figure 1. Comparison of infection efficiency of two AAV serotypes in the cochlea of cynomolgus monkeys (source: literature data collected)

- b) **OTOF cutting site study:** The cutting site between OTOF-N and OTOF-C may become another key to product design. We compared three pairs of cutting sites, introduced two pairs of cutting sites into the cochlea of young mice by using a research grade virus, applied green fluorescent protein during vector design, and took down the cochlea 10 days later to observe the fluorescence ratio in IHCs of the cochlea of mice (Figure 2). Since fluorescence can only be expressed after the splicing of OTOF-N and OTOF-C is completed, the fluorescence ratio, to some extent, represents the efficiency of OTOF-N and OTOF-C splicing. A comparison of the splicing efficiency among the three pairs of cutting sites shows that splicing efficiency is the highest at cutting site 2. Therefore, this project chooses cutting site 2 as the cutting site design of GOI.

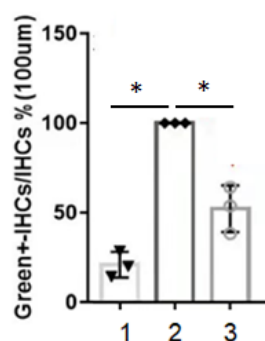


Figure 2. Comparison of in vivo splicing efficiency among three pairs of cutting sites

- c) **Promoter study:** In GOI design, promoter selection is an important aspect. Promoters determine the spatiotemporal expression characteristics of genes and are closely related to the safety and effectiveness of AAV-mediated gene therapy. Theoretically, specific promoters derived from endogenous genome sequences have advantages in safety over broad-spectrum promoters containing viral sequences (such as CMV, CAG), but specific promoters may not be as strong as broad-spectrum promoters in expression intensity. Therefore, each of these two types of promoters has its own advantages. Promoters such as CMV and CAG are widely used in the cochlea and compared extensively, and they can be highly expressed in cochlear IHCs, but the application of specific promoters in the cochlea is rarely studied.

We first selected two specific promoters and broad-spectrum promoter CMV to investigate their expression specificity and efficiency in the mouse cochlea (Figure 3). By contrast, it is found that the CMV promoter can drive efficient expression of fluorescent proteins in IHCs (indicated by red arrows in Figure 3A) and OHCs (indicated by red parenthesis in Figure 3A), and fluorescent proteins are widely expressed in other regions of the cochlea (Figure 3A). Whereas expression of green fluorescence driven by the specific promoters Promoter-1 and Promoter-2 derived from the mouse genome is localized to IHCs (indicated by red arrows in Figure 3B) and OHCs, and no expression is observed in other cells. Moreover, the expression intensity of the Promote-1-driven fluorescent protein is higher and more pronounced in the Base region.

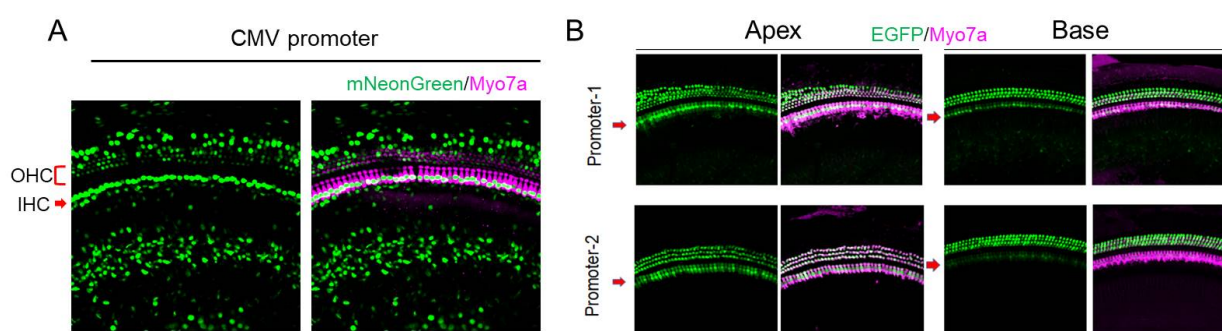


Figure 3. Comparison of efficiency between two specific promoters

Subsequently, we have selected a combination of AAV-No.2 + Promoter-1 + cutting site 2 to perform gene therapy for hearing loss in OTOV point mutation mice and conduct a comparison with the broad-spectrum promoter CMV. ABR hearing function tests show that Promoter-1 has a more sustained efficacy compared with the CMV promoter (Figure 4B), while CMV has only a short-term efficacy due to unknown reasons, and its efficacy in restoring hearing function is lost 18 days after AAV injection (Figure 4A). Therefore, combined with expression efficiency and audiometric data, the specific promoter Promoter-1 is selected for this project, which has relatively superior advantages in safety and effectiveness.

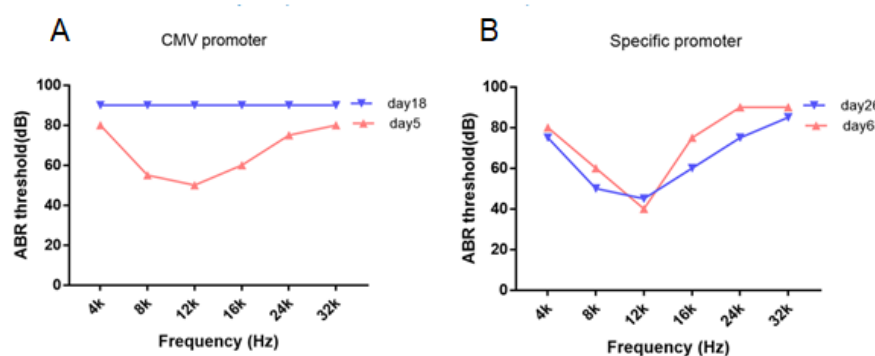


Figure 4. Comparison of efficacy between broad-spectrum promoter CMV and specific promoter Promoter-1

In summary, by using research grade viruses, we have conducted a series of studies on the composition and design of the Injection. Finally, the combination of AAV-No.2 + Promoter-1 + cutting site 2 is determined as the design of the Injection.

2) Pharmacodynamic study on GMP-Like lab-scale OTOV101N + OTOV101C Injection

We have conducted studies on ratios of the two viruses, relationship between dose and efficacy, and pharmacodynamic stability in OTOF point mutation mice to confirm the factors affecting the efficacy of OTOV101N + OTOV101C Injection. The virus used is GMP-Like lab-scale OTOV101N + OTOV101C Injection manufactured by Guangzhou PackGene Biotechnology Co., Ltd., a leading CRO company, and the titers of viruses are OTOV101N: 6×10^{13} vg/ mL, OTOV101C: 1.8×10^{13} vg/ mL, respectively. Experimental subject: OTOF point mutant mice, and virus injection time: Adult (around P30) mice.

- a) **Study on ratios of the two viruses:** We have performed pharmacodynamic studies on two ratios of OTOV-N and OTOV-C (1:1 and 2:1) and find that the 1:1 ratio is more effective in hearing restoration in OTOF point mutation adult mice and closer to hearing levels in wild-type mice. The 1:1 ratio is more effective than the 2:1 ratio in terms of both full-band hearing (Figure 5A) and Click ABR (Figure 5B). The ratio of the two viruses does affect the

therapeutic efficacy, which may be attributed to the fact that the ratio will affect the expression of the full-length human OTOF protein. Subsequent studies are therefore conducted using the 1:1 ratio.

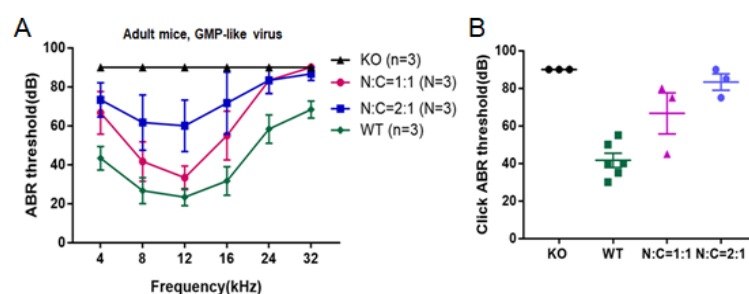


Figure 5. Effect of different ratios of OTOV-N and OTOV-C on efficacy

- b) **Study on the relationship between dose and efficacy:** Subsequently, we use a 1:1 ratio to explore the relationship between dose and efficacy in order to obtain an appropriate therapeutic dose. We select two dose groups. High-dose group: The virus stock solutions are mixed at a molar ratio of 1:1 without dilution, and the final titer is $1.4 \times 10^{13} + 1.4 \times 10^{13}$ vg/mL, the total virus amount is 5.6×10^{10} vg. Low-dose group: Accounting for 1/4 of high dose by diluting the obtained high-dose solution with the vehicle R012 solution provided by the CRO company, with the final titer of $0.35 \times 10^{13} + 0.35 \times 10^{13}$ vg/mL and the total virus amount of 1.4×10^{10} vg. The two groups of virus solutions are injected into adult (around P30) OTOF point mutation mice, and ABR hearing function tests are performed 7 days after injection. The efficacy in the high-dose group is better than that in the low-dose group in terms of both full-band hearing (Figure 6A) and Click ABR (Figure 6C). Meanwhile, the analysis of the high-dose group reveals that the hearing rehabilitation of some mice can reach the level equivalent to that of the WT mice (Figure 6B). Therefore, it is confirmed that the efficacy of high dose is better than that of low dose, though the low-dose group also shows therapeutic effect to some extent.

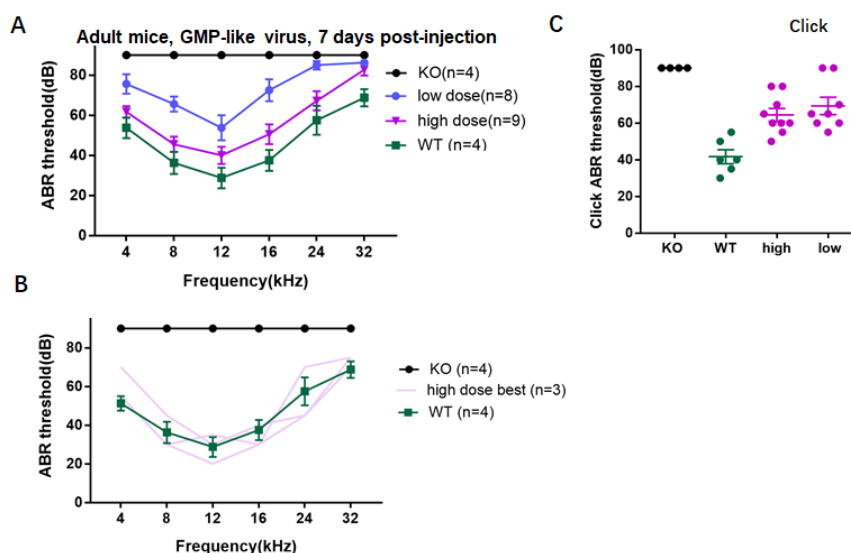


Figure 6. Relationship between dose and efficacy of GMP-Like virus

It can be found from waveform analysis at fixed frequencies that the ABR waveforms in the high-dose group are well restored in terms of both waveform and intensity (Figure 7), and even can return to the corresponding hearing level of 25 dB comparable to the WT group in the frequency band of 12 kHz (Figure 7A, 7C). The point mutation group, on the other hand, has hearing thresholds greater than 90 Db (Figure 7B).

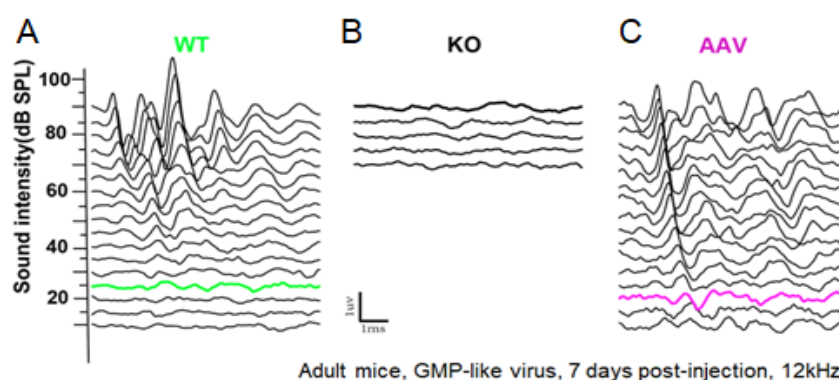


Figure 7. Comparison of hearing waveforms

- c) **Pharmacodynamic stability study:** Subsequently, we follow up and monitor the long-term efficacy of the high-dose group. We have performed audiometry tests on Day 7, Day 30, and Day 60 post-injection. Therefore, as can be seen from the effects in both full-band hearing (Figure 8A) and Click ABR (Figure 8B), the hearing rehabilitation effect of high-dose virus is stable and long-lasting.

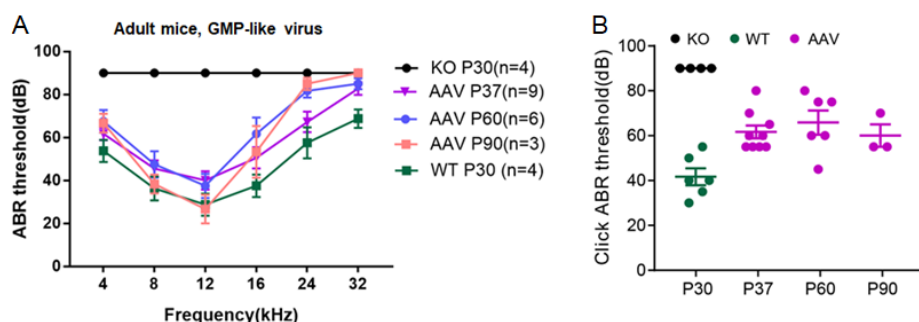


Figure 8. Monitoring of long-term efficacy of high-dose virus

In summary, by using GMP-Like lab-scale OTOV101N + OTOV101C Injection, we have confirmed the factors affecting the efficacy of OTOV101N + OTOV101C Injection. The two AAVs will be mixed in a 1:1 ratio, with a final titer of $1.4 \times 10^{13} + 1.4 \times 10^{13}$ vg/mL and a total virus amount of 5.6×10^{10} vg, which is the most suitable dose for the mouse model, and the efficacy lasts for 90 days (based on the longest observation period till now).

3) Pharmacodynamic study on GMP grade OTOV101N + OTOV101C Injection

We have commissioned the CRO company Guangzhou PackGene Biotechnology Co., Ltd. to manufacture GMP grade OTOV101N + OTOV101C Injection, which will also be used for subsequent IIT. The titer of the Injection is OTOV101N: 2.8×10^{13} vg/mL, OTOV101C: 2.8×10^{13} vg/mL. Taking the same dose setting as the high-dose group of GMP-Like lab-scale virus, we have studied the efficacy and OTOF expression of GMP-Like virus and tested the stability of product quality of different batches.

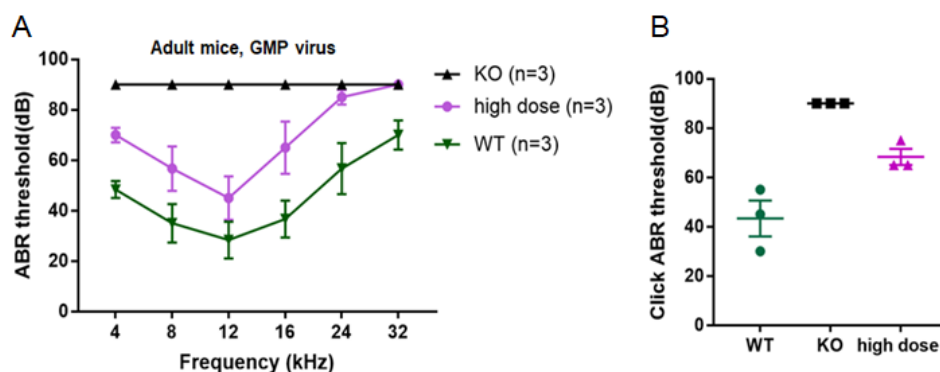


Figure 9. Efficacy of high-dose GMP virus

GMP virus (Figure 9) achieves similar effects in both full-band hearing and Click ABR as the high-dose group of GMP-Like lab-scale virus (Figure 6), indicating the stability of the virus lots. Meanwhile, we stain the cochlear anatomy injected with the virus, and the results of OTOF staining (Figure 10) show that OTOF can be highly expressed in cochlear IHCs 10 days after viral infection, with higher expression intensity in Apex and Middle regions (Figure 10A), and the statistical results also suggest that the expression ratio is higher in Apex and Middle regions, and is slightly lower in Base region (Figure 10B), which also partly explain the reason why OTOV101N + OTOV101C

Injection has a slightly poor hearing restoration effect in the high-frequency regions.

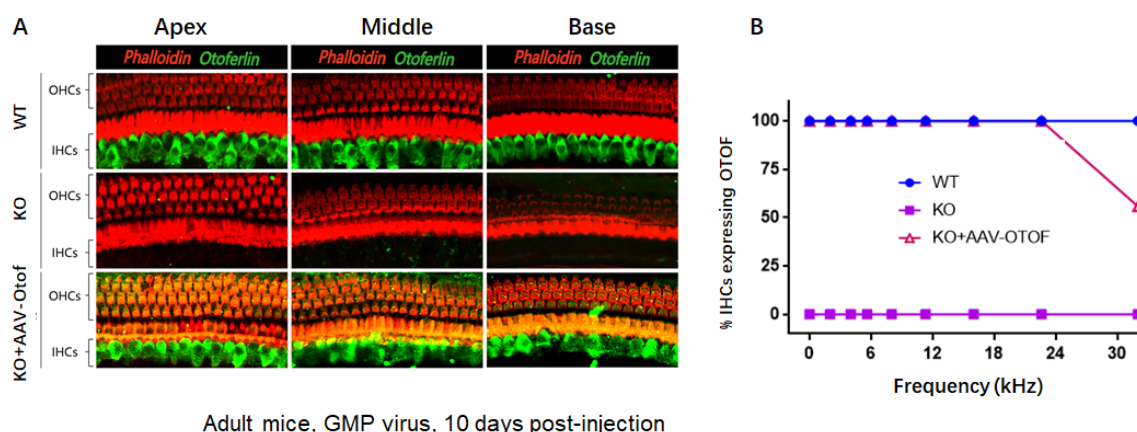


Figure 10. Expression effect of AAV-OTOF in mouse model

In summary, in the mouse model, we have validated the efficacy of GMP grade OTOV101N + OTOV101C Injection to be used in IIT.

Summary: We have elaborately investigated the composition and efficacy of OTOV101N + OTOV101C Injection from three sources. (1) By using a research grade virus, we have optimized AAV serotype, promoter and OTOF cutting site to complete the composition design of the Injection. (2) By using GMP-Like and GMP Grade Injection manufactured by the CDMO company, we have completed the determination of injection dose and virus ratio in mouse model, providing a basis for the injection dose and virus ratio in clinical trials, and the long-term efficacy observation data and OTOF expression data provide a basis for clinical trials.

1.3.2. Preclinical toxicology studies

➤ Single-dose studies in mice

Toxicological study design and grouping arrangement

Group		Control/test article	Dose (vg/ear)	Dose concentration (vg/mL)	Injection volume (μL) ^a
1	Blank control group	NA	NA	NA	NA
2	Vehicle control group	R012 solution (diluent)	0	0	2
3	Low-dose group	OTOV101N + OTOV101C	1.4E10	0.7E13	2
4	High-dose group		5.6E10	2.8E13	2

^a The route of administration is through posterior semicircular canal.

1) General observations (including injection sites)

After administration, mice in vehicle group, low-dose group and high dose group all have normal diet, normal activity and good mental status; no obvious swelling or pus is observed on the operative wound; and the wound heals well.

2) Mortality

Animals in groups 1-4 (8 mice in each group, half males and half females) are monitored for death in postoperative W1, W2, W4, M1 and M3, and the mortality of mice is statistically analyzed. It

is revealed by the analysis results that: There is no difference in mortality among the blank group, vehicle group, low-dose group and high-dose group of the same age. The surgery and the drug have no effect on long-term survival of mice.

3) Weight

Body weight of animals in groups 1-4 is measured preoperatively, and after drug injection, body weight data are collected at M1 and M2 post-injection. It is revealed by the analysis results that: The drug has no impact on the weight gain of the test animals.

4) Auditory function

In order to verify whether AAV affects hearing functions of mice, ABR tests are performed after anesthesia in mice of groups 1–4. Audiometric experiments are performed using a TDT RZ6 auditory electrophysiology workstation. During the audiometry test, the lowest stimulation volume that can induce auditory brainstem electrical signals is taken as the ABR threshold at this frequency by giving mice stimulation at different volumes (20–90 dB SPL) at different frequencies (4 kHz, 8 kHz, 12 kHz, 16 kHz, 24 kHz, 32 kHz). ABR is collected in W1 (1 week, 1w), W2 (2 weeks, 2w), M1 (1 month, 1m) and M2 (2 months, 2m) post-injection. The statistical results show that, in W1, W2, M1 and M2 post-injection, there is no significant difference in the full-band hearing threshold of ABR between mice in groups 2-4 and the controlled mice of the same age in group 1. It is revealed by the analysis results that: The surgery and the drug do not affect the normal auditory function of the test animals.

5) Behavioral studies

After 7 days of administration, there is no difference in motor functions of mice such as climbing ability, balance ability and endurance.

After 7 days of administration, there is no difference in motor functions of mice such as balance regulation ability and limb coordination ability.

After 7 days of administration, there is no difference in limb tension and exploratory ability, basic motor ability, and anxious behavior of mice.

After 7 days of administration, there is no difference in cognitive ability and memory ability of mice.

6) Blood routine examination and blood biochemistry

The results of blood routine examination show that after 7 days of the administration, the counts and morphology of various cells in the erythrocyte system, leukocyte system and platelet system of mice among the vehicle group, high-dose group and low-dose group are basically unchanged compared with those in the baseline and blank control groups of the same age.

It is revealed by the blood biochemistry results that: The amounts of various ions, carbohydrates, lipids, proteins, and various enzymes and hormones in blood as well as various metabolites in mice

remain essentially unchanged. Individual measures may vary due to the individual difference in mice.

7) Neutralizing antibody detection in the blood

It is revealed by the detection results that: Compared with the low-dose group, the high-dose group shows a higher titer of neutralizing antibodies, while neutralizing antibodies in the blank and vehicle groups are not detected (Figures 11A and 11B), and the titers of neutralizing antibodies in the high-dose and low-dose groups increase insignificantly over time from D9 to D49 post-injection (Figures 11C and 11D).

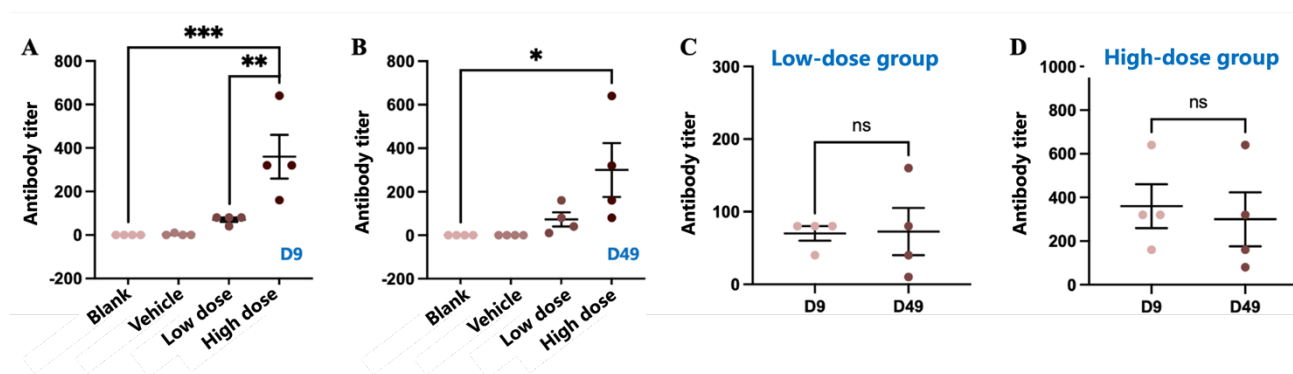


Figure 11 Neutralizing antibody titers in the blood on D9 and D49 in mice of groups 1-4

8) Histopathological examination

Low-grade inflammation (mild, I) is occasionally observed in the liver and kidneys of baseline and blank mice in group 1. Low-grade inflammation (mild, I) is occasionally observed in the liver and kidneys of mice in groups 2-4. There are no significant changes in inflammation in other organs. It can be seen from the pathological findings in W1 and M1 that, there is no substantial pathological progression of inflammation in the organs, and pathological inflammation and injuries are not significantly correlated with the administration.

In summary: In preclinical safety studies, we have performed a unilateral intracochlear administration in mice, and the results of general observation, mortality, body weight, auditory function, behavioral studies, blood routine examination, blood biochemistry, and histopathological examination of mice in vehicle group, low-dose group, and high-dose group reveal no serious injury directly related to AAV administration. Neutralizing antibody detection suggests that injection of AAV may result in elevated neutralizing antibody titers, and neutralizing antibody titer levels in the high-dose group are higher than that in the low-dose group.

1.4. Risk-benefit assessment

1.4.1. Benefit assessment

As mentioned in Section 1.1, CI itself has many shortcomings currently, such as poor tone identification, difficulty in tone processing, inability to appreciate music, and for patients with hereditary deafness due to a clear cause, gene therapy is regarded as an ideal treatment modality, that is, to radically restore or improve hearing and to approach natural sounds more closely through treatment with a single dose to deliver normal genes in replacement of the defect genes.

OTOV101N + OTOV101C Injection enables an application of AAV (adeno-associated virus) gene therapy to the inner ear for radical restoration or improving the auditory function of patients with

OTOF gene mutation-related deafness, in which OTOV101N + OTOV101C Injection is delivered into the cochlea of patients through local minimally invasive administration to achieve the purpose of restoring hearing. Moreover, gene replacement therapy has been used in proof-of-concept experiments in mouse models and is likely to shed light on patients with OTOF gene mutation-related deafness.

1.4.2. Risk assessment and handling measures

➤ AEs in animal experiments

AEs occurring in animal experiments of the study drug are detailed in Section “**1.3.2 Preclinical toxicology studies**”.

➤ Reference surgical implantation of similar cochlear implant and other relevant reports

Injection of AAV may cause the following AEs:

- 1) Infection caused by AAV injection operation^{[29][30]};
- 2) Adverse immune reactions caused by AAV itself, including hypersensitivity^[31], and elevated neutralizing antibody titers;
- 3) Adverse effects of intracranial spread of AAV on cranial nerves in the cerebral and posterior cranial nerves^[32];
- 4) Vestibular dysfunction caused by AAV as well as delivery operations^[33];
- 5) Facial nerve injury and chorda tympani nerve injury caused by AAV delivery operations^[29];
- 6) Tinnitus caused by AAV delivery operations^[33].

Corticosteroid therapy may cause the following AEs during AAV administration:

- 1) Diabetes, gastrointestinal ulcers and Cushing's syndrome are prone to occur due to large doses of glucocorticoids, which also have a strong inhibitory effect on the hypothalamus-pituitary-adrenal axis;
- 2) Concurrent infection;
- 3) Elderly patients are prone to develop hypertension with glucocorticoids;
- 4) Elderly patients, especially women after menopause, are prone to osteoporosis.

Adverse events that may occur during the cochlear surgery:

- 1) Anesthesia accidents;
- 2) Cardiac and cerebrovascular accidents;
- 3) High intraoperative blood loss requiring blood transfusion;
- 4) Temporary or permanent facial paralysis due to facial nerve injury;

- 5) Postoperative taste alteration due to chorda tympani nerve injury;
- 6) Perforation of the tympanic membrane and external auditory canal;
- 7) Damage to sigmoid sinus and dura mater;
- 8) Perilymphatic leakage and cerebrospinal fluid leakage;
- 9) Movement or removal of middle ear structures such as ossicles and chorda tympani nerves;
- 10) Inability to complete injection due to inner ear malformation or severe ossification;
- 11) Abortion of the surgery or adoption of rescue measures due to various accidental factors.

Adverse events that may occur after the cochlear surgery:

- 1) Local (incisional, middle ear, inner ear) infection, skin flap necrosis, intracranial infection;
- 2) Tinnitus, vertigo;
- 3) Inconsistency between preoperative expectations and postoperative outcomes.

Corresponding safety tests are scheduled at each visit after drug administration in this study, mainly including: Physical examination, vital signs, 12-lead ECG examination and blood routine, blood biochemistry, urine routine, coagulation function, etc. Necessary interventions may be administered on a case-by-case basis when adverse events are detected. During the study, if any adverse event occurs or any concomitant medication is used, the subject should report to the investigator in a timely manner and come to the hospital for necessary intervention if necessary. If an adverse event occurs after administration, it should be followed up until the adverse event recovers (returns to normal state or to baseline state), or until the condition is stable, or no more information is available (e.g., the subject refuses to provide more information, etc.).

The investigator will record the adverse reactions experienced by the subjects in detail and provide corresponding treatment according to the type and severity of adverse reactions. Antibiotics is given for postoperative infection. Immunosuppressive therapy is applied to adverse immune responses. Glucocorticoids and other neuroprotective measures are adopted for viral and mechanical nerve injury.

2. Study Site Information

A multi-center study, with Shandong Second Provincial General Hospital as the leading site.

3. Study Objectives

- 1) To evaluate the safety and tolerability of OTOV101N + OTOV101C Injection in patients with OTOF gene mutation-related deafness.
- 2) To evaluate the effectiveness of OTOV101N + OTOV101C Injection in patients with OTOF

gene mutation-related deafness.

4. Study Design

4.1. General principle

This is an investigator-initiated trial to evaluate the safety, tolerability, and effectiveness of OTOV101N + OTOV101C Injection in the treatment of patients with OTOF gene mutation-related deafness. The purpose of the study is to evaluate the safety, tolerability, and effectiveness of OTOV101N + OTOV101C injection in the treatment of patients with OTOF gene mutation-related deafness.

The successfully enrolled subjects will be started on glucocorticoids on D-3, and can be admitted to the hospital on D-3~D-1 in preparation for inner ear gene therapy. On the day of surgery (D0), subjects will receive the intracochlear injection of drugs for gene therapy via the round window after strict sterilization of the surgical area and general anesthesia. Adeno-associated virus (AAV) is administered to each subject at an injection volume ranging from 4.2×10^{11} vg/ear/each virus to 5.6×10^{11} vg/ear/each virus through intracochlear injection (OTOV101N + OTOV101C injection both at a titer of 2.8×10^{13} vg/mL, 15~20 μ L of each injection per ear, mixed at a 1:1 ratio for cochlear injection, with a total volume of 30~40 μ L/ear). For subjects with no cochlear implants on either side, unilateral intracochlear injection or bilateral intracochlear injection can be conducted as assessed by the investigator. For intracochlear injection, the investigators will decide if the second intracochlear injection should be conducted based on dose of the first injection by considering anatomical structure of artificial cochlea and drug loss. The timing of the second injection can be determined depending on the surgical recovery from the first injection. After receiving intracochlear injection, subjects need to stay in the hospital for observation for at least 3 days or the routine length of stay for diagnosis and treatment determined by the study site, and are allowed to return home after recovery from surgery, followed by a 5-year follow-up. The clinical study will not be concluded until the last subject is treated with OTOV101N + OTOV101C injection and completes the 5-year follow-up.

All subjects will return to the hospital (except encountering force majeure events) during the study process for safety and efficacy assessments at time points specified in the protocol (W1 $\pm$ 1d, W2 $\pm$ 3d, W3 $\pm$ 3d, M1 $\pm$ 3d, M2 $\pm$ 3d, M4 $\pm$ 6d, M6 $\pm$ 6d, M9 $\pm$ 6d, M12 $\pm$ 6d, M18 $\pm$ 14d, M24 $\pm$ 14d, M30 $\pm$ 14d, M36 $\pm$ 14d, M42 $\pm$ 14d, M48 $\pm$ 14d, M54 $\pm$ 14d, M60 $\pm$ 14d/EOS/Unscheduled).

Selection of study ear: Unilateral intracochlear injection: The ear with more severe hearing loss will be

selected as the study ear. If the hearing condition is the same in both ears, the left ear will be selected as the study ear. If one side has already received a cochlear implant, the other side is selected as the study ear. Bilateral intracochlear injection: For subjects with no cochlear implant on either side, bilateral intracochlear injection may be performed.

4.2. Sample size

Planned sample size is 25 subjects.

5. Subject Number

Subjects will be assigned a unique screening number in order of screening, which is S + two digits. For example: The screening number for the first subject is S01, for the second subject is S02, and so on for the other subjects. After successful screening, subject numbers will be assigned based on the chronological order in which subjects are enrolled. Subject numbers are Y + two digits. For example: The subject number for the first subject is Y01, for the second subject is Y02, and so on for the other subjects.

6. Study Subject

Patients aged 1 year and older with severe or greater deafness due to OTOF gene mutations who have remaining inner hair cells.

6.1. Inclusion criteria

Subjects must meet all of the following inclusion criteria to be enrolled in the study:

- 1) Age ≥ 1 year at the time of signing the ICF; male or female;
- 2) Diagnosis of OTOF related deafness is established as per the following criteria:
 - a) Hearing loss confirmed by auditory testing and auditory brainstem response (ABR) test (test reports within 1 month before the signing of ICF are acceptable);
 - b) Presence of homozygous or compound heterozygous mutations in the OTOF gene confirmed by genetic testing;
- 3) Auditory testing: Bilateral severe ($65 \text{ dB} \leq \text{hearing threshold} < 80 \text{ dB}$) or profound ($80 \text{ dB} \leq \text{hearing threshold} < 95 \text{ dB}$) or complete (hearing threshold $\geq 95 \text{ dB}$) hearing loss; (if ABR test results are “not produced”, subjects with bilateral hearing threshold $< 65 \text{ dB}$ can be enrolled as judged by the investigator);
- 4) Test results of vital signs, physical examination, laboratory tests (blood routine examination, blood biochemistry and urine routine examination, coagulation function, etc.), 12-lead ECGs

and other tests are all normal, or the abnormality is judged by the investigators as having no clinical significance;

- 5) Subjects and their guardians sign the ICF.

6.2. Exclusion criteria

Patients meeting any of the following criteria should not be enrolled:

- 1) Patients who have previously experienced serious allergic reactions to any drug or its components in this study ($\geq$ Grade 3 Severe AE as per NCI-CTCAE v 5.0);
- 2) Any prior gene therapy or blood neutralizing antibody level greater than 1:128;
- 3) Patients with systemic diseases or having received relevant treatment that may affect hearing or surgical operation;
- 4) Patients intolerable to general anesthesia;
- 5) Patients with inner ear malformation;
- 6) Bilateral cochlear implantation, or history of major inner ear surgery (as judged by the investigator) (except unilateral cochlear implantation);
- 7) Patients with other mutations in deafness gene that affect judgment of OTOF gene therapy effect;
- 8) Patients with Meniere's disease;
- 9) Patients who are routinely treated with ototoxic drugs for other diseases;
- 10) Patients with congenital deafness due to the presence of other non-genetic factors associated with hearing loss at birth;
- 11) Subjects who are receiving or are likely to receive immunosuppressive therapy other than the study drug of this study;
- 12) Subjects with hypersensitivity or intolerance to glucocorticoids;
- 13) History of malignancy or meningitis;
- 14) Subjects with persistent or active infection, positive HBsAg results and HBV DNA titer in peripheral blood higher than the lower limit of detection, positive HCV antibody test results and HCV RNA titer in peripheral blood higher than the lower limit of detection, positive HIV antibody test results or other immunodeficiency diseases, and positive syphilis test results;

- 15) Subjects of childbearing potential who refuse to use effective contraception (hormonal or barrier contraception or abstinence) from signing the informed consent to 12 months after injection of AAV;
- 16) Female subjects of childbearing potential with positive blood pregnancy test results or who are pregnant or lactating;
- 17) Subjects who have participated in any other clinical trial within 4 weeks prior to the first dose and have received administration or treatment (including drug and device clinical trials, excluding non-interventional studies);
- 18) Subjects who are unwilling or unable to comply with this protocol;
- 19) Subjects who, in the opinion of the investigators, are unable to participate in the study or to complete the subsequent study due to any medical conditions.

6.3. Withdrawal criteria

- 1) Subjects wrongly included, judged as not meeting inclusion criteria or meeting exclusion criteria by the investigator;
- 2) Subjects failing to receive gene therapy after enrollment;
- 3) Occurrence of an AE, which is considered by the investigators, cooperative institution or medical monitor as having caused unacceptable consequences or risks to the subjects;
- 4) The safety of the subject, as considered by the investigators, is compromised at any time during the trial;
- 5) Subjects with poor compliance who, as assessed by the investigators, are not suitable to continue this study;
- 6) Other reasons that the investigators consider it inappropriate for the subject to continue the trial;
- 7) Withdrawal of informed consent at any time by the subject or his/her guardian (if any);
- 8) Subjects lost to follow-up.

When a subject withdraws prematurely from the study, the investigator should ask the subject whether he/she is willing to continue follow-up visits and whether he/she agrees to record the discussion results in the medical history and eCRF. Where a subject is lost to follow-up, the investigator should make every effort to reach out to the subject or his/her relative, get acquainted with the reason for his/her withdrawal from the study, complete the last assessment, and record the reason

for the subject's withdrawal from the study.

Where a subject withdraws from the study due to an AE, the AE should be followed up until the subject returns to normal or the baseline level, or the status of the subject is stable or the subject is lost to follow-up, and corresponding records should be made.

6.4. Termination criteria

- 1) Under the premise of fully protecting the rights and interests and safety of subjects, the investigators or cooperative institution demands termination;
- 2) Regulatory authorities or the Ethics Committee order termination of the study due to reasons such as significant safety risks of the study drug;
- 3) Other reasons that the cooperative institution or the investigator considers inappropriate to continue the study.

The study may continue once the abovementioned issues about drug safety, protocol compliance, and data quality leading to study suspension have been resolved and agreement from the cooperative institution, the Ethics Committee, or the National Medical Products Administration (NMPA) have been obtained.

7. Study Drug

Name	OTOV101N + OTOV101C Injection
Titer of study drug	2.8×10^{13} vg/ mL
Route of administration	Intracochlear injection via round window membrane
Transport and storage conditions	Not above -60°C
Drug provider	Suzhou Otovia Therapeutics Co., Ltd.

7.1. Management of study drug

7.1.1. Packaging and labeling of study drug

Study drugs are packaged and labeled by Suzhou Otovia Therapeutics Co., Ltd.

Label content includes, but is not limited to: Clinical trial number, product name, strength, batch number, usage, drug storage conditions, manufacturing date, shelf life, manufacturer, words of “only for clinical study use” and entrusting unit.

7.1.2. Receipt, distribution, and storage of study drug

The study drug and its injector will be provided free of charge by Suzhou Otovia Therapeutics Co., Ltd. after being approved by the investigator and the Ethics Committee according to the requirements of the study protocol and distributed to the clinical study site as planned. When receiving the study drug and its injector, the clinical study site should sign the receipt form of the drug and its injector, then verify and sign for receipt on the spot in duplicate, with the clinical study site and the cooperative institution holding one copy each.

The study drug should be kept, managed, counted, distributed, and recovered by a person in charge of the clinical study site, and should be used within the framework of the clinical study as required by the protocol. Besides, the person is also responsible for documenting the receipt, distribution and return of the study drug as required by the cooperative unit. All unused or used drugs and packaging will be recovered or destroyed according to the *Study Drug Management Manual* by the investigator during the study/at the end of the study.

8. Study Procedure

8.1. Screening period

Screening is within 60 days prior to treatment. Before the subject receives gene therapy, a detailed medical history is collected for each subject, and examinations on hearing in both ears, and the outer, middle, and inner ears are performed, including: Behavioral audiometry, tympanogram, DPOAE, cochlear microphonic potential measurement, ABR, ASSR examination, and cranial MRI/CT examination. Prior to gene therapy, venous blood is drawn from subjects and tested for neutralizing antibody levels. If a subject is unable to provide the genetic test report for deafness or the genetic test report for deafness does not contain parental verification, venous blood will be collected from the subject for molecular diagnosis to confirm the type of gene defect, and venous blood will be collected from the parents of the subject for pedigree verification. After checking the inclusion and exclusion criteria, the investigator decides whether the subject can be enrolled.

Relevant examinations and assessments during the screening period should be completed before administration, which are as follows:

- 1) Signing of informed consent form;
- 2) Demographic collection: Including gender, age, date of birth and ethnicity of subjects;
- 3) Medical history: Past medical history and treatment history, inner ear surgery history, allergy history, and tumor history should be recorded.

- 4) Vital signs: Vital signs, including body temperature, blood pressure, pulse, and respiratory rate, are measured after subjects have rested quietly for at least 5 min. The same position should be maintained at each visit as far as possible;
- 5) Physical examination: Including general condition, skin mucosa, lymph nodes, head, neck, chest, abdomen, spine, limbs and neurological examination. In addition to general examination, neurological examination focuses on trigeminal nerve, facial nerve, vagus nerve, glossopharyngeal nerve, and accessory nerve functions as well as taste function;
- 6) Body weight measurement;
- 7) ECG: Examinations are performed after subjects have rested quietly in a recumbent position for at least 5 min; including heart rate, PR interval, QRS interval, QT interval, and QTc interval.
- 8) Blood pregnancy test: This test is only performed for female subjects of childbearing age who is capable of childbearing;
- 9) Virological test: HBsAg test, HCV test, HIV antibody test, and syphilis antibody test. For HBsAg-positive subjects, HBV-DNA testing is required. HCV antibody-positive subjects should receive HCV-RNA test. Test results from our hospital or other hospitals within 60 days before administration are acceptable;
- 10) Blood routine and urine routine; See Attachment II for details of test items;
- 11) Blood biochemistry: See Attachment II for details of test items;
- 12) Coagulation function test: See Attachment II for details of test items;
- 13) Brain MRI/CT: For patients with cochlear implants, cranial MRI is not required if there are previous cranial MRI results (no time window is required). Cranial CT is required if there are no previous cranial MRI results. And cranial MRI is required for patients without cochlear implants;
- 14) Auditory testing: Behavioral audiometry, tympanogram, DPOAE, CM, ABR, ASSR examination;
- 15) Venous blood draw: Neutralizing antibody detection;
- 16) Genetic testing: If a subject is unable to provide the genetic test report for deafness or the genetic test report for deafness does not contain parental verification, venous blood will be collected from the subject for molecular diagnosis to confirm the type of gene defect, and

venous blood will be collected from the parents of the subject for pedigree verification.

17) Recording of concomitant medications.

8.2. Gene therapy

Following enrollment, eligible subjects receive oral prednisone 0.5 mg/kg body weight/day or equivalent of other corticosteroids starting 3 days prior to gene therapy. The dose is increased to 1 mg/kg body weight/day from the day of surgery to W1, decreased to 0.5 mg/kg body weight/day at W2~W3, decreased to 0.25 mg/kg body weight/day at W4~W5 and decreased to 0.125 mg/kg body weight/day at W6~W8. Then, the drug is discontinued. Maximum prednisone dose is no more than 40 mg/day. The actual dose administered is assessed by the investigator based on subjects' actual condition.

During the period from three days before to the day before gene therapy (D-3~D-1), subjects should be admitted to the hospital in preparation for the surgery, and re-check various preoperative test items, including: Vital signs, physical examination, ECG, laboratory indicators, blood pregnancy. Subjects then receive unilateral cochlear injection for gene therapy under general anesthesia on the day of gene therapy (D0). For subjects with no cochlear implants on either side, unilateral intracochlear injection or bilateral intracochlear injection may be performed as assessed by the investigator. According to the wishes of subjects and their guardians, funding will be provided for this project to complete unilateral cochlear implantation, but the cochlear implant is not within the scope of observation and follow-up of this project (except for efficacy data such as auditory testing). Prophylactic antibiotics should be administered until postoperative day 3.

Second injection operation: The investigators will decide if the second intracochlear injection should be conducted based on dose of the first injection by considering anatomical structure of artificial cochlea and drug loss. The timing of the second injection will be decided by recovery status of the first injection. Auditory testing is required before the second injection (the test results within 1 month before surgery is acceptable). The second injection is performed on D-3~D-1 and D0 visits according to the flow chart. The follow-up visit (D1~M60 ± 14d/EOS) is performed according to the time of the second surgery.

D-3~D-1

- 1) Vital signs;
- 2) Physical examination;

- 3) Body weight measurement;
- 4) ECG;
- 5) Blood pregnancy test: This test is only performed for female subjects of childbearing age who is capable of childbearing;
- 6) Blood routine and urine routine; See Attachment II for details of test items;
- 7) Blood biochemistry: See Attachment II for details of test items;
- 8) Coagulation function test: See Attachment II for details of test items;
- 9) Glucocorticoid dispensing (glucocorticoid dispensing starts 3 days prior to receiving gene therapy);
- 10) Oral glucocorticoid administration (glucocorticoids are given at the established dose specified by the protocol 3 days prior to receiving gene therapy);
- 11) Recording of concomitant medications.

D0 (Day of surgery)

- 1) Vital signs;
- 2) Prophylactic antibiotic administration;
- 3) Glucocorticoid dispensing;
- 4) Oral glucocorticoid administration;
- 5) Intracochlear injection;
- 6) Recording of adverse events;
- 7) Recording of concomitant medications.

8.3. Post-treatment periodic examinations

Subjects are examined for hearing along with recovery after intracochlear injection surgery, the presence of incisional and intracranial infections, tinnitus and vestibular dysfunction, and cranial nerve dysfunction, particularly trigeminal, facial, vagal, glossopharyngeal, and accessory nerves, and adverse event reports are recorded.

At scheduled visits, venous blood is drawn for neutralizing antibody level testing and AAV level testing to assess safety.

D1

- 1) Vital signs;
- 2) Physical examination;

- 3) Venous blood draw: AAV testing;
- 4) Prophylactic antibiotic administration;
- 5) Oral glucocorticoid administration;
- 6) Recording of adverse events;
- 7) Recording of concomitant medications.

D3

- 1) Vital signs;
- 2) Physical examination;
- 3) ECG;
- 4) Blood routine and urine routine; See Attachment II for details of test items;
- 5) Blood biochemistry: See Attachment II for details of test items;
- 6) Coagulation function test: See Attachment II for details of test items;
- 7) Venous blood draw: Neutralizing antibody and AAV testing;
- 8) Prophylactic antibiotic administration;
- 9) Glucocorticoid dispensing;
- 10) Oral glucocorticoid administration;
- 11) Recording of adverse events;
- 12) Recording of concomitant medications.

W1 ± 1d, W2 ± 3d, W3 ± 3d

- 1) Vital signs;
- 2) Physical examination;
- 3) Body weight measurement: At W2 only (patient self-measurement results are acceptable);
- 4) Blood routine and urine routine; See Attachment II for details of test items;
- 5) Blood biochemistry: See Attachment II for details of test items;
- 6) Auditory testing: Behavioral audiometry, tympanogram, DPOAE, CM, ABR, ASSR examination, at W2 ± 3d only;
- 7) Venous blood draw: Neutralizing antibody and AAV testing;
- 8) Glucocorticoid dispensing;
- 9) Oral glucocorticoid administration;
- 10) Recording of adverse events;

11) Recording of concomitant medications.

M1 ± 3d, M2 ± 3d, M4 ± 6d, M6 ± 6d, M9 ± 6d

- 1) Vital signs;
- 2) Physical examination;
- 3) Body weight measurement: At W4 (i.e., M1), W6 only, and if necessary (patient self-measurement results are acceptable);
- 4) Blood routine and urine routine; See Attachment II for details of test items;
- 5) Blood biochemistry: See Attachment II for details of test items;
- 6) Auditory testing: Behavioral audiometry, tympanogram, DPOAE, CM, ABR, ASSR examination;
- 7) Venous blood draw: Neutralizing antibody and AAV testing;
- 8) Glucocorticoid dispensing, at M1 ± 3d only;
- 9) Oral glucocorticoid administration; to M2, i.e., W8;
- 10) Recording of adverse events;
- 11) Recording of concomitant medications.

M12 ± 6d/EOS/Unscheduled

- 1) Vital signs;
- 2) Physical examination;
- 3) ECG;
- 4) Blood pregnancy test: This test is only performed for female subjects of childbearing age who is capable of childbearing;
- 5) Blood routine and urine routine;
- 6) Blood biochemistry;
- 7) Coagulation function test;
- 8) Auditory testing: Behavioral audiometry, tympanogram, DPOAE, CM, ABR, ASSR examination;
- 9) Venous blood draw: Neutralizing antibody and AAV testing;
- 10) Recording of adverse events;
- 11) Recording of concomitant medications.

EOS visit: Occurred between D1-M12 visits, hearing tests are performed as per requirements of M12 visit; occurred between M18-M60 visits, hearing tests are performed as per requirements of M18-M60 visits.

M18 ± 14d, M24 ± 14d, M30 ± 14d, M36 ± 14d, M42 ± 14d, M48 ± 14d, M54 ± 14d, M60 ± 14d

- 1) Vital signs
- 2) Physical examination;
- 3) ECG;
- 4) Blood routine and urine routine;
- 5) Blood biochemistry;
- 6) Auditory testing: Behavioral audiometry/pure tone audiometry, ABR examination;
- 7) Recording of adverse events;
- 8) Recording of concomitant medications.

During the study, the auditory testing (behavioral audiometry, tympanogram, DPOAE, CM, ABR, ASSR examinations) during the screening period is mandatory, and at subsequent visits, it may be decided whether to increase/decrease the auditory testing visits and increase/decrease the auditory test items based on the subjects' willingness of cooperation and the assessment of investigators

During the study, subjects will receive regular post-treatment visits and no longer receive scheduled periodic examinations after the end of the study, but the clinical study team will also establish contact with each subject and arrange follow-up visits according to each subject's personal circumstances to ensure that subjects will receive appropriate medical measures after the end of the study.

9. Concomitant medication

➤ Concomitant medication

- 1) Prednisone or equivalent oral corticosteroids are administered for the first 3 days of receiving gene therapy until W8. Maximum prednisone dose is no more than 40 mg/day.
- 2) To prevent infection, prophylactic antibiotics should be administered until postoperative day 3, and the specific dosage regimen and duration of administration should be based on the study institution's practice.

➤ **Medications and therapies used concomitantly during the study as appropriate**

- 1) Chronic medications required prior to enrollment for concomitant diseases that, in the judgment of the investigators, will not pose a risk of ototoxicity;
- 2) Symptomatic treatment for drug toxicities.

➤ **Prohibited medications during the study**

During the study, concomitant medications other than those specified in the protocol and those that may be used concomitantly as appropriate, are prohibited.

When a subject needs to use other drugs during the study, he/she should consult the responsible investigator beforehand, and inform the responsible investigator truthfully afterwards even if he/she fails to reach the investigator due to special reasons.

10. Evaluation indicators

10.1. Safety evaluation indicators

All AEs throughout the study are assessed and graded according to NCI-CTCAE v 5.0. Safety evaluation indicators include:

- 1) Incidence and severity of AEs and their correlation with the study drug;
- 2) Physical examination, including cranial nerve testing;
- 3) Laboratory test indicators (blood routine, urine routine, blood biochemistry, coagulation function, etc.), vital signs and ECG results examination;
- 4) Neutralizing antibodies detection and AAV testing in peripheral blood.

10.2. Efficacy evaluation indicators

According to the degree of hearing restoration of subjects, the investigator should test the hearing of subjects according to the required follow-up time points, and the content of the test mainly include:

- 1) Hearing assessment: Behavioral audiometry, ABR, ASSR, distortion product otoacoustic emission (DPOAE) response;
- 2) Cochlear microphonic (CM) potentials;
- 3) Tympanogram examination.

11. Adverse Events and Serious Adverse Events

11.1. Adverse events

11.1.1. Definition of adverse events

An AE is defined as any adverse medical events occurred in a subject after receiving the study

drug, which can manifest as symptoms and signs, disease, or laboratory abnormalities and does not necessarily have a causal relationship with the study drug.

Adverse medical events should be recorded as AEs from the time the subject receives the first dose of the study drug until 12 months of follow-up or until another treatment regimen is initiated, whichever comes earlier.

11.1.2. Collection and recording of adverse events

AEs should be recorded faithfully during the study. Their types, severity, occurrence time, duration, treatment measures, treatment process and outcomes should be recorded in detail, and their correlation with the study drug should be evaluated based on comprehensive consideration of comorbidities and concomitant medications.

Any change determined by the investigator to meet the definition of an AE should be recorded in the AE section of the eCRF. Medical documentation of AEs should be recorded in source documents.

11.1.3. Criteria for determining the severity of adverse events

The severity of adverse events is recorded using the National Cancer Institute's Common Terminology Criteria for Adverse Events CTCAE v 5.0 and graded from 1 - 5, with AEs not listed in the table of CTCAE v 5.0 graded from 1 - 5 according to the criteria in the table below.

Criteria for determining the severity of adverse events

Grade	CTCAE status
1	Mild: Asymptomatic or mild symptoms; clinical or diagnostic observations only; intervention not indicated.
2	Moderate: Minimal, local, or noninvasive intervention indicated; limiting age-appropriate instrumental activities of daily living (ADL) *.
3	Severe or medically significant but not immediately life-threatening: Hospitalization or prolongation of hospitalization indicated; disabling, limiting self-care ADL **.
4	Life-threatening consequences: Urgent intervention indicated.
5	Death related to adverse events.

CTCAE=Common Terminology Criteria for Adverse Events

*: Instrumental ADL refers to preparing meals, shopping for groceries or clothes, using the telephone, managing money, etc.

**: Self-care ADL refers to bathing, dressing and undressing, feeding self, using the toilet, taking medications, and not bedridden.

Note the distinction between severe adverse events and serious adverse events. Severe is used to describe intensity and not necessarily SAEs. For example, headache may be severe in intensity, but should not be listed as an SAE unless it meets the criteria for SAEs.

11.1.4. Criteria for determining the relationship between adverse events and study drug

The investigator evaluates the relationship between AEs and the study drug according to the classifications provided in the table below. Reactions definitely related, probably related, possibly related, and possibly unrelated to the study drug (note: possibly unrelated reactions may require reasonable evidence to support the existence of a possible causal relationship to be classified as adverse reactions) are classified as adverse reactions, and the incidence of adverse reactions should be counted.

5-grade classification	Criteria for judgment
Definitely related	Consistent with the type of reaction known to occur with the suspected drug; consistent with a reasonable temporal sequence after administration; the adverse reaction is relieved or disappears after dose reduction or drug withdrawal, and reappears after re-administration.
Probably related	Consistent with the type of reaction known to occur with the suspected drug; consistent with a reasonable temporal sequence after administration; the adverse reaction is relieved or disappears after dose reduction or drug withdrawal, but the subject's clinical status or other reasons may also cause the reaction.
Possibly related	Consistent with the type of reaction known to occur with the suspected drug; consistent with a reasonable temporal sequence after administration; the adverse reaction is relieved or insignificant after dose reduction or drug withdrawal, but the subject's clinical status or other reasons may explain the reaction.
Possibly unrelated	Less consistent with the type of reaction known to occur with the suspected drug; less consistent with a reasonable temporal sequence after administration; the subject's clinical status or other reasons may also cause the reaction.
Definitely unrelated	Inconsistent with the type of reaction known to occur with the suspected drug; inconsistent with a reasonable temporal sequence after administration; the adverse reaction remains unchanged after dose reduction or drug withdrawal; the subject's clinical status or other reasons may also explain the reaction, the reaction is relieved or disappears when clinical symptoms or other causes are ruled out.

11.1.5. Follow-up of adverse events

All adverse events (including laboratory abnormalities) must be followed up until one of the following conditions is achieved:

- 1) The event is resolved;
- 2) The event remains stable;
- 3) The event does not require further follow-up as judged by the investigator;
- 4) The event returns to baseline;

- 5) The event can be attributed to a drug other than the study drug or unrelated to the study conduct;
- 6) When more information is not available (the subject refuses to provide more information or there is evidence that the subject remains lost to follow-up after best efforts have been made).

11.2. Serious adverse events

11.2.1. Definition of serious adverse events

Serious adverse events (SAE): Adverse medical events result in death, life-threatening conditions, persistent or significant disability or incapacity, requirement for inpatient hospitalization or prolongation of existing hospitalization, or a congenital anomaly or birth defect in a subject after receiving the study drug.

An adverse event is considered serious when it meets any one or more of the following conditions:

- 1) Results in death;
- 2) Is life-threatening (the occurrence of an adverse event places the subject at immediate risk of death and it does not include an adverse event that, had it occurred in a more severe form, might have caused death, for example: drug-induced hepatitis without hepatic failure);
- 3) Results in inpatient hospitalization or prolongation of existing hospitalization; if a subject has a medical condition or illness that existed prior to enrollment in the study, for which admission to the hospital for treatment and/or surgery is planned prior to the start of the study or during the course of the study, but does not deteriorate in an unexpected manner during the study, it is not an SAE but should be clearly documented in the medical record;
- 4) Results in congenital anomaly or birth defect;
- 5) Results in permanent or significant disability or incapacity;
- 6) Is an other important medical event: It may not be immediately life-threatening, result in death or hospitalization, but is usually considered serious if medical measures are required to prevent one of the above outcomes. For example, important treatment in the emergency room or allergic bronchospasm at home, cachexia or convulsion without hospitalization, drug dependence or addiction.

Note:

- Hospitalization/prolongation of hospitalization also includes intra-hospital transport to an emergency/intensive care unit (e.g., from pediatric department to internal medicine

department, from internal medicine department to coronary care unit, from neurology department to tuberculosis unit, etc.).

- Diagnostic and therapeutic noninvasive and invasive procedures, such as surgery, should not be reported as adverse events. However, the disease treated by the procedure should be reported as an adverse event if it meets the definition of an adverse event. For example, acute appendicitis occurring during the adverse event reporting period should be reported as an adverse event, and appendectomy performed to treat the disease should be documented as a treatment for that adverse event.

The following situations will not be handled as SAEs:

- Emergency room visit;
- Admission for observation within 24 hours;
- Admission for routine outpatient examination (hospitalization for less than 24 hours);
- Minor surgical procedures (e.g., ophthalmic laser surgery) performed in a hospital or center;
- Conditions described in the protocol: Hospitalization for administration, hospitalization for completion of protocol-required procedures;
- Hospitalization for social reasons: for example, the patient is hospitalized due to lack of care;
- Hospitalization for surgery on an agreed date prior to study entry must be documented in the source records and medical records (considered an SAE if the condition worsens and requires earlier surgery);
- Hospitalization in rehabilitation facilities, nursing homes;
- Routine hospitalization for physical examinations;
- Disease progression of indication should not be considered an adverse event. However, symptoms and signs of disease progression may be reported as SAEs if they meet the criteria for SAEs;
- Death itself is a consequence and is not considered an SAE. The primary cause of death (primary AE leading to death) should be recorded and reported as an SAE. “Death” is reported as the consequence of the respective AE. If no exact cause of death can be reported, the death itself can be reported as an SAE. Subject death due to disease progression should also be reported as an SAE.

11.2.2. Reporting of serious adverse events

In case of an SAE, regardless of its relationship with the study drug, the investigator should take appropriate treatment measures in a timely manner in addition to completing the SAE report. In addition to serious adverse events that do not require immediate reporting as specified in the study protocol or other documents (such as the Investigator's Brochure), the investigator should report all serious adverse events in writing to the cooperative institution within 24 hours of being informed of the occurrence of SAEs to the extent possible, and should subsequently provide a detailed written follow-up report in a timely manner. The identification code of the subject in the clinical study should be indicated in the serious adverse event report and follow-up report, rather than the subject's real name, citizen identification number, address and other identity information. Adverse events and abnormal laboratory values specified in the study protocol that are important for safety evaluation should be reported to the cooperative institution according to the requirements and time limit specified in the study protocol.

For the reporting of death events, the investigator should provide other required data, such as autopsy report and final medical report, to the collaborating institution and the Ethics Committee.

11.3. Suspected unexpected serious adverse reactions

11.3.1. Definition of SUSAR

Suspected unexpected serious adverse reactions (SUSARs) refer to suspected and unexpected serious adverse reactions whose nature and severity of clinical manifestations exceed the information available in the Investigator's Brochure of the study drug, the package inserts of the marketed drug or the summary of product characteristics.

Suspected refers to that the relationship with the study drug cannot be excluded. See the definition of correlation in "11.1.4 Criteria for judging the relationship between adverse events and the study drug" for details.

The definition of serious adverse events is described in "11.2.1 Definition of serious adverse events".

Unexpected adverse reactions refer to adverse reactions whose nature, severity, consequences or frequency are different from the expected risks described in the current relevant information of the study drug (such as the Investigator's Brochure, etc.). As a primary document, the Investigator's Brochure provides reference safety information to determine whether an adverse reaction is expected or unexpected.

11.3.2. Reporting of SUSAR

The cooperative institution should determine the expectedness and correlation of SAEs received during the clinical study of the drug, and SUSARs determined as related to the study drug should be reported to the drug regulatory authorities and competent health authorities within the specified time limit (7/15 days) according to relevant regulations. The cooperative institution should report SUSARs to all investigators participating in the clinical study, clinical study institutions and the Ethics Committee in an expedited manner.

The investigator should report SUSARs provided by the cooperative institution to the Ethics Committee.

11.4. Pregnancy

If the subject (or sexual partner of the subject) becomes pregnant within 12 months after administration, this event should be reported to the cooperative institution and the Ethics Committee as soon as possible after being informed of it. Once a pregnancy event occurs during the study, the investigator should decide whether to terminate the subject's continued participation in the clinical study as soon as possible. The investigator should communicate scientifically and rigorously with the subject based on the medication information, to inform her/him of the possible effects and risks of the study drug to the pregnant woman and fetus, and to leave it to the subject's discretion to terminate or to continue the pregnancy.

The investigator should complete the initial report on the pregnancy report form and report to the cooperative institution within 24 hours after being informed of the pregnancy of the subject (or sexual partner of the subject).

If the subject (or sexual partner of the subject) decides to terminate the pregnancy, the investigator should complete the corresponding section of the pregnancy report form and report to the cooperative institution within 24 hours after being informed of the termination.

If the subject (or sexual partner of the subject) decides to continue the pregnancy, follow-up of the pregnancy is required every 3 months until the subject gives birth and the outcome of the pregnancy is known. Moreover, the investigator should complete the follow-up report on the pregnancy report form within 24 hours of each pregnancy follow-up visit, report to the cooperative institution, and report to the Ethics Committee of the hospital if necessary.

Pregnancy itself is not considered an AE or SAE. If the outcome of the pregnancy meets the criteria for serious adverse events (e.g., spontaneous abortion, stillbirth, neonatal death, or congenital

anomaly including an aborted fetus, stillbirth, or neonatal death), the investigator should report it according to the reporting procedure for serious adverse events.

All neonatal deaths occurring within one month of birth should be reported as serious adverse events regardless of the cause of death. In addition, any infant death after one month of birth should also be reported as long as the investigator considers the death to be possibly related to the study drug.

12. Data Management

12.1. Source data and source documents

Source data refers to all information recorded in original records or certified copies of the clinical study, including clinical findings, observations, and records of other relevant activities required for the reconstruction and evaluation of the clinical study.

Source documents refer to original records, documents and data generated in the clinical study, such as hospital medical records, medical images, laboratory records, memoranda, subject diaries or evaluation forms, dispensing records, data automatically recorded by instruments, microfiches, photographic negatives, magnetic media, X-ray films, subject files, and documents and records related to the clinical study maintained by pharmacies, laboratories and medical technology departments, including certified copies, etc. Source documents include source data and may exist as paper or electronic carriers.

12.2. Database creation

In this study, remote data entry of eCRF will be used to collect data from the clinical study. The data manager designs the database and tests it to ensure that it is accurate.

12.3. Data entry and modification

Data entry and modification are completed by the investigator. Data should be sourced from original documents such as original record forms and laboratory test reports and should be consistent with original documents. Any observation and test results in the trial should be timely, correctly, completely, clearly, normatively and authentically entered into the form or database. The data manager is responsible for reviewing and managing the entered data. For data queries, the data manager will send corresponding queries to the investigator, and the investigator should respond to queries sent by the data manager promptly. The data manager may re-query if necessary.

12.4. Confidentiality plan for study data

The study data is confidential, and all study members are required to keep the study data

confidential. They should not inform members outside the study group of the study data without the permission of the principal investigator, should not transfer the study data to other institutions without the permission of the hospital, and should not transfer the study data involving human genetic resources to foreign institutions or domestic institutions with foreign investment without the approval of the Office for Human Genetic Resources Management of China, except for the publication of the study results that normally meet the regulatory requirements.

12.5. Database verification

Methods of data verification during data management include: Data logic check, manual verification, medical verification, statistical pre-analysis verification and other stages. Data queries will be presented in the EDC system in the form of electronic queries to be answered by the study site. If the answer satisfies the requirement, the query will be closed. If a data query remains unresolved or new query is generated after updating the database according to the answer of the previous data query, it needs to be re-answered by the investigator or by CRC in confirmation with the investigator. The above process will be repeated until all data in the database is confirmed to be correct.

12.6. Database locking

The data manager should write the data review report according to the study protocol, data review criteria and database. The project manager should hold a data review meeting attended by the cooperative institution, principal investigator, statistician and data manager to review the data, and the representatives of all participating parties should sign the data review resolution. After the cooperative institution approves to lock the data, the data manager organizes to perform the data locking. Locked data will be submitted to the statistician for statistical analysis.

Data will be locked when the following conditions are met.

- 1) All data have been collected and stored;
- 2) All codes have been verified and confirmed;
- 3) All data queries have been resolved (including queries raised by data review);
- 4) Data review has been completed;
- 5) Source data verification has been completed;
- 6) Serious adverse event reconciliation has been completed;
- 7) External data reconciliation has been completed;
- 8) All investigators' signatures have been obtained;

- 9) Analyzable cases have been defined and maintained in the final analysis database.

12.7. Statistical analysis data sets

Full Analysis Set (FAS): All subjects who have received the study drug; the FAS is mainly used for the analysis of demographic and baseline data, subject distribution and protocol violations.

Safety Analysis Set (SS): All subjects who have received the study drug and have at least one post-treatment safety evaluation data; SS is primarily used for the analysis of safety data.

Effectiveness Analysis Set (EAS): All subjects who have received the study drug and completed at least one post-baseline hearing function test. EAS is primarily used for analysis of efficacy data.

12.8. Overall analysis plan

Baseline data will be summarized by treatment group in the safety analysis set. For continuous variables, the mean, standard deviation, quartiles, extreme values will be estimated. For categorical variables, frequency and percentage will be estimated.

Baseline data will be summarized in the Safety Analysis Set. For continuous variables, the statistical description of mean, standard deviation, quartiles, maximum and minimum values will be performed. For categorical variables, frequency and corresponding percentage will be described.

Safety analysis will be performed in the Safety Analysis Set and summarized by clinical visit. Safety analyses include otologic examinations, laboratory tests, and other reported otologic or systemic AEs. AEs will be summarized by type and frequency.

Efficacy analyses will be performed in the EAS, where quantitative efficacy indicators will be summarized by clinical visit for the treated ear and contralateral ear, and trends in change from baseline will be calculated, including means, standard deviations, and 95% confidence intervals for changes. Qualitative efficacy indicators will be summarized by clinical visit for the treated ear and contralateral ear, and changes from baseline will be described using a cross-tabulation table before and after treatment. This study is an exploratory study and no formal hypothesis testing will be performed.

13. Study Management

13.1. Ethics

13.1.1. Ethical requirements

The study protocol should be reviewed and approved by the Ethics Committee of the hospital before implementation. The cooperative institution and investigator should provide relevant documents to the Ethics Committee during the review. This study must comply with the *Declaration*

of Helsinki, Good Clinical Practice (2020) promulgated by the NMPA in conjunction with the National Health Commission, and relevant regulations. During the study, any amendment to the study protocol must be approved or filed by the Ethics Committee. It is the responsibility of the investigator to inform the Ethics Committee that the study has been completed at the end of the study in accordance with the relevant requirements of the Ethics Committee.

13.1.2. Informed consent

The informed consent form (ICF) (along with the study protocol) must be reviewed and approved by the Ethics Committee.

It is the responsibility of the investigator or a person designated by him/her to explain the content of the informed consent form to the subject in a manner and wording understandable to the subject, so that it can be easily understood by the subject. When the investigator obtains any new information that may affect the subject's continued participation in the study, he/she should inform the subject in a timely manner and make corresponding records.

The final text of the informed consent form and other information provided to the subject should include the following content: Overview of the clinical study, study objective, study treatment, study procedures to be followed by the subject (including invasive medical procedures), obligations of the subject, experimental content involved in the clinical study, risks or inconveniences that may be caused to the subject by the study, the compensation and treatment available to the subject in the event of a study-related injury, the compensation available to the subject for participating in the clinical study; and that subjects' participation in the study is voluntary; they may refuse to participate or have the right to withdraw from the study at any time and at any stage of the study without discrimination or retaliation, and his/her medical treatment and rights and interests will not be affected.

Prior to signing the informed consent form, the investigator or his/her designated study staff should give the subject sufficient time and opportunity to understand the details of the clinical study. The subject and the investigator performing the informed consent should sign and date the informed consent form separately. The investigators and the subject should each retain 1 copy of the informed consent form. If important new information involving the study drug is found, the informed consent form must be obtained from the subject again after it is revised in writing and submitted to the Ethics Committee for approval.

13.1.3. Subject confidentiality

It is the responsibility of the investigator to maintain the anonymity of subjects. Subjects should be identified only by capital letters, numbers, and/or codes and not by their names on CRFs or other documents. The investigator must maintain the subject enrollment log documenting the subject code, name, and home address. The investigator must maintain strict confidentiality on documents that can identify the subject.

13.2. Responsibilities assumed by each party

Responsibilities assumed by each party are carried out in accordance with the GCP (2020) or a signed contract or agreement.

13.3. Protocol deviations

The investigator or a person designated by him/her should record and explain any deviation from the approved protocol, fill in the clinical study protocol deviation report, and submit it to the Ethics Committee according to the requirements of the committee. Once the clinical research associate (CRA) determines the occurrence of any deviation from the clinical study protocol, he/she should communicate with the investigator promptly and take appropriate measures to prevent the recurrence of deviation.

If the investigator or medical institution has serious problems or non-compliance problems after dissuasion, the cooperative institution should terminate the continued participation of the investigator or medical institution in the clinical study and promptly report to the drug regulatory authority in writing.

13.4. Training

According to the GCP principles, the CRA should have the qualification recognized by the cooperative institution. He/she should conduct training for the investigator on the study protocol before the start of the clinical study, familiarize with and understand the content of the clinical study protocol, master the GCP principles, unify the recording method and criteria for judgment, and strictly implement the protocol.

13.5. Standardized operation

The cooperative institution should comply with the regulations of current ICH/GCP and *Provisions for Drug Registration* and the study drug meet the relevant requirements of Good Manufacturing Practice. They are subject to strict quality inspections.

Laboratory tests are performed by the study site according to SOP.

Laboratory test methods and quality control at the study site should be unified. The clinical laboratory of the study site must carry out internal quality control as required and obtain the certificate of conformity for quality evaluation of the National Center for Clinical Laboratories.

13.6. Quality control and assurance

- 1) SOPs should be established for all study processes.
- 2) Qualification of study sites: Clinical study sites are sites identified by the NMPA as having clinical conditions to conduct the clinical study.
- 3) Qualification of study staff: Study staff must be trained in clinical studies. The investigator and other study staff are trained on GCP and the study protocol.
- 4) Laboratory quality control measures: Laboratories should establish standard operating procedures and quality control procedures for experimental observation indicators.
- 5) Study monitoring: The CRA has received training on GCP and study protocol, and has expertise required for clinical trial monitoring such as medical and pharmacy knowledge. The CRA will perform regular monitoring during the progress of the study. The CRA must ensure that the clinical facilities of the study site meet the requirements, and ensure that the investigator follows the study protocol and records the study results accurately. During each monitoring, the clinical research associate must review eCRFs and original documents, and also check the storage conditions and the use records of the drug.
- 6) Study audits: According to the requirements of GCP, study sites must also be subject to audits by auditors designated by the cooperative institution. Auditors should systematically inspect the activities and documents related to the clinical study to evaluate whether the study is conducted in accordance with the study protocol, standard operating procedures and relevant regulatory requirements, and whether the study data are recorded in a timely, authentic, accurate and complete manner. Audits should be performed by persons not directly involved in the clinical study.

13.6.1. Monitoring

During clinical study, a monitoring plan and SOPs for monitoring should be developed. The monitoring plan should comply with relevant laws and regulations, consist of the monitoring strategy, monitoring responsibilities for all parties in the study, monitoring methods, and reasons for applying different monitoring methods, and emphasize the protection of the rights and interests of subjects, the

guarantee of the authenticity of data, and the assurance of coping with all kinds of risks in the clinical study.

The specific content of the monitoring includes:

- 1) Prior to the study, confirm that the study site is equipped with appropriate conditions, including staffing and training, that the laboratory is well equipped and functioning with various study-related examination conditions, that a sufficient number of subjects are estimated to be available, and that staff participating the study are familiar with the requirements of the study protocol.
- 2) Verify that the study drug is supplied, stored, distributed and recovered according to relevant procedures, and make corresponding records;
- 3) Monitor the implementation of the study protocol by the investigator during the study, verify that the investigator performs the responsibilities specified in the study protocol and contract, and whether these responsibilities are delegated to unauthorized personnel; ensure that the investigator receives the latest version of the Investigator's Brochure, all study-related documents, and supplies necessary for the study, and perform them in accordance with requirements of relevant laws and regulations;
- 4) Confirm the eligibility of enrolled subjects, and report the enrollment rate and progress of the clinical study;
- 5) Confirm the accuracy and completeness of CRF entry, as well as its consistency with source documents; confirm follow-ups, studies and examinations not performed by the investigator, and whether corrections have been made to errors and omissions and documented in the CRF; verify whether the withdrawal and loss to follow-up of enrolled subjects have been documented and explained in the CRF; ensure that corrections, additions or deletions are made by the investigator or an authorized person, and signed and dated by the person who makes the modification, with justifications for modification provided if necessary;
- 6) Confirm that all adverse events have been reported within the specified time limit in accordance with relevant laws and regulations, the study protocol and requirements of the Ethics Committee and the cooperative institution;
- 7) Communicate with the investigator in a timely manner about deviations from the study protocol, standard operating procedures, and requirements of relevant laws and regulations,

- and take appropriate measures to prevent their recurrence;
- 8) Assist the investigator to make necessary notification and application, and report study data and results to the cooperative institution;
 - 9) Complete a written monitoring report after each monitoring, stating the date and time of monitoring, name of the CRA, name of the investigator and other staff to whom the CRA is encountered. The cooperative institution should review and follow up on the issues in the monitoring report and document them for retention.

13.6.2. Audit and inspection

The quality management department (representative) of the cooperative institution may conduct audits at the study site. Audits include, but are not limited to, compliance with the protocol, supply of the study drug, retention of required documents, informed consent process, and comparison of eCRFs with source documents. The investigator is obliged to cooperate with the audit. In addition, the NMPA and provincial administration where the study site is located may conduct the inspection, and the investigator should immediately notify the cooperative institution of any inspection.

The investigator/institution should uniformly allow the auditor or inspector to access directly to all relevant documents, and the investigator and his/her staff should make time available to the auditor or inspector to discuss and examine any findings. Audits and inspections may be conducted at any time during or after the completion of the study.

13.7. Recording and storage of study data

According to GCP, for clinical studies not used to apply for drug registration, essential documents should be retained for at least 5 years after termination of the clinical study.

13.8. Confidentiality and publication of study results

All information about this study (not limited to the following documents: protocol, Investigator's Brochure) must be kept strictly confidential. The investigator must recognize that the scientific or medical information obtained from this study may be of commercial value to the cooperative institution. The investigator should maintain the confidentiality of information and data related to this study, and should consult with and obtain written consent from the cooperative institution in advance if he/she intends to publish information related to this study or the conclusions drawn from the study. In order to protect its own rights and interests, the cooperative institution may require the investigator not to publish any information about the study until the product under study is approved for marketing.

The cooperative institution has the right to release or publish information or data related to this study, or submit it to drug regulatory authorities. The cooperative institution should obtain the investigator's consent if the investigator's name is to appear in the content of the publication, journal, or advertisement.

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Attachment I: Contraceptive measures and definition of women of childbearing age

- **Definition of women of childbearing age**

Women who are not of childbearing age are defined as postmenopausal women and premenopausal women who have been surgically sterilized. Postmenopausal is defined as cessation of menses for ≥ 12 months without alternative medical measures. A follicle-stimulating hormone (FSH) test can be performed in indeterminate subjects with $\text{FSH} > 40 \text{ MIU/mL}$ confirming menopause. Sterilization includes bilateral tubal ligation, bilateral oophorectomy, or hysterectomy performed 12 weeks prior to the first dose.

Women of childbearing age are defined as those who have not undergone surgical sterilization and are anatomically and physiologically capable of becoming pregnant following menarche and prior to menopause.

Attachment II: Laboratory tests (blood routine, urine routine, blood biochemistry and coagulation function)

Blood biochemistry Alanine aminotransferase (ALT) Aspartate aminotransferase (AST) Alkaline phosphatase (ALP) Total bilirubin (TBIL) Direct bilirubin (DBIL) Total cholesterol (CH) Triglycerides (TG) High density lipoprotein cholesterol (HDL) Low density lipoprotein cholesterol (HDL) Lipoprotein (a) (LPa) Total protein (TP) Albumin (ALB) Globulin (GLO) Albumin/Globulin (A/G) Blood glucose (GLU) Urea (UREA) Creatinine (CREA) Uric acid (UA) Calcium (Ca ²⁺), phosphorus (P), potassium (K ⁺), sodium (Na ⁺) and chloride (Cl ⁻)	Blood routine tests White blood cells (WBC) Lymphocyte count (LYMPH#) Monocytes (MONO#) Neutrophil count (NEUT#) Eosinophils (EOS#) Basophils (BASO#) Lymphocyte percentage (LYMPH%) Monocyte percentage (MONO%) Neutrophil percentage (NEUT%) Hematocrit (HCT) Eosinophil percentage (EOS%) Basophil percentage (BASO%) Red blood cells (RBC) Hemoglobin (HGB) Platelets (PLT) Urine routine test Urobilinogen (URO) Urine bilirubin (BIL) Urine ketone body (KET) Urinary occult blood (BLD) Urine protein (PRO) Urine nitrite (NIT) Glucose in urine (GLU) Specific gravity of urine (SG) Urine pH (PH) Red blood cells in urine (RBC) White blood cells in urine (WBC)
Coagulation function tests Prothrombin time (PT) Activated partial thromboplastin time (APTT) Thrombin time (TT) Fibrinogen (FIB)	

Note: The above test items should be subject to the study site.

Protocol Amendment History

Summary of the major modifications to the trial protocol between version 1.0 and 1.5, aiming to enhance its rationality and practical operability while considering the clinical risks and benefits for the subjects involved in the trial.

Major modifications	Reasons
1) Deleted “brain MRI” from safety evaluation indicators; brain MRI/CT should be performed only during the screening period, and whether to perform MRI/CT during subsequent follow-up should be decided at the discretion of the investigator; cases where MRI or CT should be performed are made clear; 2) Auditory testing: Revised auditory test items, deleted acoustic reflex, added tympanogram, and added behavioral audiometry and ASSR test; deleted auditory test in W3; added auditory test visit at the discretion of the investigator, added or removed auditory test items at the discretion of the investigator. 3) AAV testing: Added AAV testing on postoperative day 1. 4) Route of administration: Deleted single injection; 5) Increased the screening period to 60 days. 6) Genetic testing: Added pedigree verification. 7) Added body weight measurement on D-3 ~ D-1. 8) Changed from single center to multi-center. 9) Changed unilateral injection to allow bilateral injection as assessed by investigators. 10) Inclusion criteria 1: Adjusted “Age ≥ 3 years” to “Age ≥ 1 year” 11) Inclusion criteria 2b: Deleted “test report within 1 month before informed consent is acceptable” 12) Changed the planned sample size from “5~6” to “25” subjects.	1) Due to the local study drug delivery method, MRI detection after treatment is not necessary; 2) To comprehensively evaluate the inner ear structure and auditory function after surgery, relevant indicator detections have been added. Since follow-ups are scheduled at both 2 weeks and 4 weeks post-operation, the follow-up in the third week has been removed to avoid excessive follow-up activities for patients. However, considering the potential risks and benefits of this treatment, investigators can decide whether to add or remove follow-up content and adjust the follow-up time. 3) Toxicological studies in cynomolgus monkeys showed that AAV-<i>OTOF</i> translocates from the inner ear to the blood within 24 hours. Thus, a day1 post-operative whole-blood AAV detection was added to the clinical protocol to comprehensively elucidate the temporal changes in the biodistribution of AAV-<i>OTOF</i>, thereby facilitating a more in-depth understanding of its pharmacokinetic profile and potential safety implications. 4) The investigators will decide if the second intracochlear injection should be conducted based on dose of the first injection by considering anatomical structure of artificial cochlea and drug loss. The timing of the second injection will be decided by recovery status from the first injection. 5) In order to fully evaluate whether the patient meets the enrollment criteria. 6) More stringent enrollment criteria have been implemented to ensure that patients truly have DFNB9 caused by <i>OTOF</i> mutations. 7) Body weight has been incorporated into the protocol as one of the safety assessment indicators. 8) In order to enroll more patients. 9) Since binaural hearing recovery improves sound localization, in additions, safety and efficacy were confirmed, we expanded the trial to bilateral injection. 10) To understand the potential benefits for toddlers. 11) Longer-term test report is accepted for DFNB9 diagnosis. 12) To explore and expand the clinical benefits for more patients, including the adult patient, the number of participants has

13) Dosage regimen: Adjust injection volume.	been increased due to the well-tolerance of AAV- <i>OTOF</i> and robust hearing improvement.
14) Increased follow-up period to 5 years.	13) To balance the clinical risk and benefit.
15) Nab testing: Conduct NAb testing until the results turn negative.	14) To assess the long-term safety.
	15) NAb test is unnecessary after response turned negative.