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Supplementary information

Oncolytic virus VG161 in refractory hepatocellular carcinoma

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Supplementary Information (SI)

Oncolytic Virus VG161 in Refractory Hepatocellular Carcinoma

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Supplementary Table 1| Characteristics of each patient

ID	Sex	Age	HCC or ICC	BOR (RECIST)	BOR (mRECIST)	Tumor Evaluation Method	Pre-treatment Biopsy Pathology[3]	Post-treatment Biopsy Pathology[4]
A1-101	Man	70	HCC	SD	SD	Enhanced CT	Yes	No
A2-102	Man	66	HCC	SD	PR	Enhanced CT	Yes	No
A3-104	Man	73	HCC	SD	SD	Enhanced CT	Yes	No
A3-108	Man	54	HCC	PD	PD	Enhanced CT	Yes	No
A4-109	Man	48	HCC	SD	SD	Enhanced CT	Yes	No
A4-110	Man	43	HCC	PD	PD	Enhanced CT	Yes	No
A5-119	Man	55	HCC	PD	PD	Enhanced CT	Yes	No
A5-120	Man	57	HCC	PD	PD	Enhanced CT	Yes	No
B0-126	Man	52	HCC	SD	SD	Enhanced CT	Yes	No
B0-128	Woman	71	HCC	SD	SD	Enhanced CT	Yes	No
B0-129	Man	67	HCC	PR	PR	Enhanced CT	Yes	Yes (Injection Lesion + Non-injection Lesion)
B0-130	Man	62	HCC	PD	PD	Enhanced CT	Yes	No
B0-132	Man	62	HCC	SD	SD	Enhanced CT	Yes	No
B0-134	Man	50	HCC	PD	PD	Enhanced CT	Yes	No
B0-136	Man	39	HCC	PD	PD	Enhanced CT	Yes	No

Supplementary Table 1| Characteristics of each patient

ID	Sex	Age	HCC or ICC	BOR (RECIST)	BOR (mRECIST)	Tumor Evaluation Method	Pre-treatment Biopsy Pathology[3]	Post-treatment Biopsy Pathology[4]
B0-137	Man	63	HCC	PR	PR	Enhanced MRI	Yes	No
B0-143	Man	61	HCC	SD	SD	Enhanced CT	Yes	No
B0-148	Man	52	HCC	SD	SD	Enhanced CT	Yes	No
B0-150	Man	58	HCC	SD	SD	Enhanced MRI	Yes	No
B0-151	Man	62	HCC	PD	PD	Enhanced MRI	Yes	No
B0-154	Man	47	HCC	PD	PD	Enhanced CT	Yes	No
B0-155	Man	65	HCC	SD	SD	Enhanced CT	Yes	No
B0-159	Man	48	HCC	PR	PR	Enhanced CT	Yes	No
B0-160	Man	72	HCC	NA[1]	NA[1]	NA[1]	Yes	No
B0-161	Man	66	HCC	NA[1]	NA[1]	NA[1]	Yes	No
B0-162	Man	72	HCC	PD	PD	Enhanced CT	Yes	No
B0-163	Man	66	HCC	SD	PR	Enhanced CT	Yes	No
B0-164	Man	56	HCC	SD	SD	Enhanced CT	Yes	No
B0-165	Man	57	HCC	SD	SD	Enhanced CT	Yes	No
B0-170	Man	59	HCC	PD	PD	Enhanced CT	Yes	No
B0-301	Woman	42	HCC	SD	SD	Enhanced CT	Yes	No

Supplementary Table 1| Characteristics of each patient

ID	Sex	Age	HCC or ICC	BOR (RECIST)	BOR (mRECIST)	Tumor Evaluation Method	Pre-treatment Biopsy Pathology[3]	Post-treatment Biopsy Pathology[4]
B0-302	Woman	43	HCC	PD	PD	Enhanced MRI	Yes	No
B0-304	Man	49	HCC	NA[1]	NA[1]	NA[1]	Yes	No
B0-171	Man	59	HCC	SD	PR	Enhanced MRI	Yes	No
B0-173	Man	43	HCC	PD	PD	Non-contrast CT + Enhanced MRI	Yes	No
B0-176	Man	69	HCC	SD	SD	Enhanced CT	Yes	No
B0-179	Man	61	HCC	SD	PR	Enhanced CT	Yes	Yes (Injection Lesion)
B0-180	Man	58	HCC	SD	SD	Enhanced CT	Yes	Yes (Injection Lesion)
B0-181	Man	58	HCC	SD	SD	Enhanced CT	Yes	Yes (Injection Lesion)
B0-183	Man	70	HCC	SD	SD	Enhanced CT	Yes	Yes (Injection Lesion)
A3-105	Woman	58	ICC	PR	NA[2]	Enhanced CT	Yes	No
A4-202	Woman	71	ICC	PD	NA[2]	Enhanced CT	Yes	No
A5-205	Man	62	ICC	PD	NA[2]	Enhanced MRI	Yes	No
B0-133	Woman	50	ICC	PD	NA[2]	Enhanced CT	Yes	No

Abbreviation: HCC, hepatocellular carcinoma; ICC, intrahepatic cholangiocarcinoma; SD, stable disease; PD, progressive disease; PR, partial response; MRI, magnetic resonance imaging; CT, computed tomography.

Supplementary Table 1| Characteristics of each patient

ID	Sex	Age	HCC or ICC	BOR (RECIST)	BOR (mRECIST)	Tumor Evaluation Method	Pre-treatment Biopsy Pathology[3]	Post-treatment Biopsy Pathology[4]

[1]Withdrawal of Informed Consent; [2]Tumor evaluations for ICC patients are not reported in this study; [3]Pre-treatment biopsy pathology was used to confirm the tumor type; [4]Post-treatment biopsy pathology was used solely for scientific analyses, such as evaluating the immune microenvironment, and not for tumor assessment. Tumor evaluation in this study was based on imaging examinations. Additionally, all biopsy procedures were conducted with signed informed consent from the patients.

Supplementary Table 2|Treatment-related adverse events (Dose-group based)

	Dose escalation (PFU)					Dose expansion (PFU)	Total
Adverse event-Term	**1.0×10^8 (n=1)** **n (%)**	**2.0×10^8 (n=1)** **n (%)**	**3.0×10^8 (n=3)** **n (%)**	**4.0×10^8 (n=3)** **n (%)**	**5.0×10^8 (n=3)** **n (%)**	**3.0×10^8 (n=33)** **n (%)**	**(n=44)** **n (%)**
Pyrexia	1 (100.0%)	1 (100.0%)	3 (100.0%)	3 (100.0%)	3 (100.0%)	27 (81.8%)	38 (86.4%)
Lymphocyte count decreased	1 (100.0%)	1 (100.0%)	3 (100.0%)	2 (66.7%)	2 (66.7%)	28 (84.8%)	37 (84.1%)
White blood cell count decreased	1 (100.0%)	1 (100.0%)	3 (100.0%)	3 (100.0%)	2 (66.7%)	21 (63.6%)	31 (70.5%)
Hypoalbuminemia	1 (100.0%)	0	2 (66.7%)	2 (66.7%)	1 (33.3%)	23 (69.7%)	29 (65.9%)
Anemia	0	1 (100.0%)	0	3 (100.0%)	3 (100.0%)	22 (66.7%)	29 (65.9%)
Platelet count decreased	1 (100.0%)	0	3 (100.0%)	3 (100.0%)	1 (33.3%)	15 (45.5%)	23 (52.3%)
Neutrophil count decreased	1 (100.0%)	1 (100.0%)	3 (100.0%)	2 (66.7%)	2 (66.7%)	14 (42.4%)	23 (52.3%)
Hypokalemia	0	0	1 (33.3%)	0	2 (66.7%)	8 (24.2%)	11 (25.0%)
Blood bilirubin increased	0	0	1 (33.3%)	2 (66.7%)	1 (33.3%)	7 (21.2%)	11 (25.0%)
Aspartate aminotransferase increased	0	0	1 (33.3%)	1 (33.3%)	2 (66.7%)	4 (12.1%)	8 (18.2%)
Vomiting	1 (100.0%)	0	1 (33.3%)	0	0	4 (12.1%)	6 (13.6%)
Nausea	1 (100.0%)	1 (100.0%)	1 (33.3%)	0	0	1 (3.0%)	4 (9.1%)
Chills	1 (100.0%)	0	1 (33.3%)	1 (33.3%)	1 (33.3%)	0	4 (9.1%)

Supplementary Table 2|Treatment-related adverse events (Dose-group based)

	Dose escalation (PFU)					Dose expansion (PFU)	Total
Adverse event-Term	**1.0×10^8 (n=1)** **n (%)**	**2.0×10^8 (n=1)** **n (%)**	**3.0×10^8 (n=3)** **n (%)**	**4.0×10^8 (n=3)** **n (%)**	**5.0×10^8 (n=3)** **n (%)**	**3.0×10^8 (n=33)** **n (%)**	**(n=44)** **n (%)**
Abdominal pain	0	0	1 (33.3%)	1 (33.3%)	0	1 (3.0%)	3 (6.8%)
Headache	0	0	0	1 (33.3%)	0	2 (6.1%)	3 (6.8%)
Weakness	0	0	0	0	1 (33.3%)	2 (6.1%)	3 (6.8%)
Gamma-glutamyltransferase increased	0	0	0	1 (33.3%)	1 (33.3%)	0	2 (4.5%)
Alanine aminotransferase increased	0	0	1 (33.3%)	1 (33.3%)	0	0	2 (4.5%)
Proteinuria	0	0	0	1 (33.3%)	0	1 (3.0%)	2 (4.5%)
Diarrhea	0	1 (100.0%)	0	0	0	1 (3.0%)	2 (4.5%)
Cold and heat intolerance	1 (100.0%)	1 (100.0%)	0	0	0	0	2 (4.5%)
Blood pressure increased	0	0	0	0	1 (33.3%)	1 (3.0%)	2 (4.5%)
Mesenteric panniculitis	0	0	1 (33.3%)	0	0	0	1 (2.3%)
Musculoskeletal pain	0	0	0	0	0	1 (3.0%)	1 (2.3%)
Elevated thyroxine	0	0	0	0	0	1 (3.0%)	1 (2.3%)
Increased procalcitonin	0	0	0	1 (33.3%)	0	0	1 (2.3%)

Supplementary Table 2|Treatment-related adverse events (Dose-group based)

	Dose escalation (PFU)					Dose expansion (PFU)	Total
Adverse event-Term	**1.0×10^8 (n=1)** **n (%)**	**2.0×10^8 (n=1)** **n (%)**	**3.0×10^8 (n=3)** **n (%)**	**4.0×10^8 (n=3)** **n (%)**	**5.0×10^8 (n=3)** **n (%)**	**3.0×10^8 (n=33)** **n (%)**	**(n=44)** **n (%)**
Facial paralysis	0	0	1 (33.3%)	0	0	0	1 (2.3%)
Decreased appetite	1 (100.0%)	0	0	0	0	0	1 (2.3%)
Cytokine release syndrome	0	0	1 (33.3%)	0	0	0	1 (2.3%)
Chest discomfort	0	0	0	0	0	1 (3.0%)	1 (2.3%)

Supplementary Table 3| Survival comparison between VG161-C102 and historical control

Trial or registration number (phase)	Population	Arm	Patients' number	Median OS	6m OS (%)	12m OS (%)
RESORCE[1] (III)	Sorafenib failed or intolerance	Regorafenib	379	10.6	70.0*	45.0*
		Placebo	194	7.8	59.0*	29.0*
KEYNOTE-240[2] (III)	Sorafenib failed or intolerance	Pembrolizumab	278	13.9	77.0*	54.0*
		Placebo	135	10.6	73.0*	48.0*
NCT01908426[3] (III)	≥1L chemo/target failed or intolerance	Cabozantinib	470	10.2	72.0	46.0
		Placebo	237	8.0	61.0	34.0
AHELP[4] (III)	≥1L chemo/target failed or intolerance	Apartinib	261	8.7	70.0	36.8
		Placebo	132	6.8	56.1	28.9
NCT02989922[5] (II/III)	≥1L chemo/target failed or intolerance	Camrelizumab	109	14.2	75.9	59.6
		(2 wks or 3 wks)	108	13.2	73.0	52.2
VG161-C102	Entire cohort	VG-161	37	12.4	79.0	50.5
VG161-C102 failed after 2L group	Failed after 2L		34	9.4	80.2	47.8
VG161-C102 PreCPI>3 m group	Failed after 2L		22	17.3	89.6	67.7
VG161-C102 PST group	Failed after 2L		11	20.1	90.0	70.0

Abbreviation: OS, overall survival; wks, weeks; PreCPI, previous checkpoint inhibitor; PST, post systemic therapy after VG161.

[1]Bruix, J., et al. Lancet 389, 56-66 (2017); [2]Finn, R.S., et al. J Clin Oncol 38, 193-202 (2020); [3]Abou-Alfa, G.K., et al. N Engl J Med 379, 54-63 (2018); [4]Qin, S., et al. Lancet Gastroenterol Hepatol 6, 559-568 (2021); [5]Qin, S., et al. Lancet Oncol 21, 571-580 (2020).

*The data is extrapolated from the Kaplan-Meier curve results presented in the article.

Supplementary Table 4| Demographic and baseline characteristics of subgroups

	PreCPI ≤ 3 months n=12 (%)	**PreCPI > 3 months n=22 (%)**	***P* value**	**PST n=11 (%)**	**non-PST n=23 (%)**	***P* value**
Age (years)			0.982[1]			0.768[1]
Mean ± SD	57.2±7.7	57.1±10.3		57.8±7.4	56.8±10.3	
Median	58	59		58	58	
Minimum, maximum	47, 73	39, 72		43, 70	39, 73	
Gender (%)			0.412[2]			0.451[2]
Male	12 (100%)	20 (90.9%)		11 (100%)	21 (91.3%)	
female.	0 (0%)	2 (9.1%)		0 (0%)	2 (8.7%)	
ECOG (%)			0.113[2]			0.306[2]
0	1 (8.3%)	7 (31.8%)		3 (27.3%)	5 (21.7%)	
1	11 (91.7%)	15 (68.2%)		8 (72.7%)	18 (78.3%)	
HBV positive	11 (91.7%)	19 (86.4%)	0.399[2]	10 (90.9%)	20 (87.0%)	0.420[2]
Prior treatments						
Tumor-related surgery	9 (75.0%)	19 (86.4%)	0.252[2]	8 (72.7%)	20 (87.0%)	0.217[2]
Radiotherapy	3 (25.0%)	7 (31.8%)	0.286[2]	2 (18.2%)	8 (34.8%)	0.206[2]
TACE	11 (91.7%)	20 (90.9%)	0.463[2]	11 (100%)	20 (87.0%)	0.296[2]
Systemic therapy						

Supplementary Table 4| Demographic and baseline characteristics of subgroups

	PreCPI ≤ 3 months n=12 (%)	**PreCPI > 3 months n=22 (%)**	***P* value**	**PST n=11 (%)**	**non-PST n=23 (%)**	***P* value**
Chemotherapy	0 (100%)	0 (100%)	NA	0 (100%)	0 (100%)	NA
Targeted therapy	12 (100%)	22 (100%)	NA	11 (100%)	22 (100%)	NA
Immunotherapy	12 (100%)	22 (100%)	NA	11 (100%)	22 (100%)	NA
Median number of prior systemic therapies (Minimum, maximum)	3 (2, 5)	3 (2, 5)	0.535[3]	2 (2, 5)	3 (2, 5)	0.161[3]

Abbreviation: PreCPI, previous checkpoint inhibitor; PST, post systemic therapy; SD, standard deviation; ECOG, eastern cooperative oncology group; HBV, hepatitis B virus; TACE, transcatheter arterial chemoembolization.

[1] T test; [2] Fisher exact test; [3] Wilcoxon test

Clinical Study Protocol

Protocol Title:	**An Open-Label, Phase I Dose-escalation, Clinical Trial To Evaluate the Safety, Tolerability, Pharmacokinetic Profile and Preliminary Efficacy of VG161 in the Treatment of Patients with Advanced Primary Liver Cancer**
Protocol No.:	**VG161-C102**
Protocol Version and Date of the Protocol:	**11-Nov-2022, version 2.0**
Study Phase:	**Phase I**
Sponsor:	**CNBG-Virogin Biotech (Shanghai) Ltd.** Room 602, Building 6, No. 955 Liuxiang Road, Nanxiang Town, Jiading District, Shanghai, P.R. China

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Clinical Trial Protocol Consent Form

I have read this clinical trial protocol (Protocol No.: VG161-C102, Version: 2.0) and agree to conduct the clinical trial following all the provisions of this protocol, current regulations, and the ethical principles of the Declaration of Helsinki.

Principal Investigator Name (Print):

Signature/Date of Principal Investigator:

DD.MM.YYYY

Name/Address of the Site:

Clinical Trial Protocol Consent Form

I have read this clinical trial protocol (Protocol No.: VG161-C102, Version: 2.0) and agree to conduct the clinical trial following all the provisions of this protocol, current regulations, and the ethical principles of the Declaration of Helsinki.

Name of Sponsor Representative (Print): Sun Changcheng Title: Clinical Director

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1 Protocol synopsis

Protocol #	VG161-C102
Study title	An open-label phase I dose-escalation clinical trial to evaluate the safety, tolerability, pharmacokinetic profile and preliminary efficacy of VG161 in patients with advanced primary liver cancer
Version no./date	Version 2.0, 11-Nov-2022
Sponsor	Company Name: CNBG Virogin Biotech (Shanghai) Co., Ltd. Project director: Changcheng Sun Contact Phone #:021-69152787
Leading site	Research institution: The First Affiliated Hospital, Zhejiang University School of Medicine Principal Investigator: Professor Liang Tingbo
Clinical phase	Phase I
Objectives	**Period 1. Single-agent dose-escalation period** Primary Objectives: To evaluate the safety and tolerability of single and multiple intratumoral injections of VG161 under ultrasound guidance to treat patients with primary liver cancer who have failed standard treatments, explore the maximum tolerated dose (MTD) of VG161, and determine the recommended administration dose. Secondary Objectives: 1) To evaluate the pharmacokinetic (P.K.) profile and virus excretion of single and multiple intratumoral injections of VG161; 2) To evaluate the preliminary efficacy of VG161 monotherapy; 3) To monitor changes in immune measures related to the pharmacodynamics of VG161. **Period 2. Monotherapy extension period** Primary Objectives: To evaluate the efficacy of multiple intratumoral injections of VG161 under ultrasound or CT guidance in treating patients with advanced primary liver cancer. The main observation measure is the objective response rate (ORR). Secondary Objectives: 1) To evaluate the secondary observation measure of the efficacy of VG161; 2) To further evaluate the safety of VG161; 3) To monitor changes in immune measures related to the pharmacodynamics of VG161.
Methodology	An open-label, phase I clinical trial

Number of Participants (Planned)	Period 1. Single-agent dose-escalation period: about 14 cases (Actual enrolment 11 cases) Period 2. Monotherapy extension period: 30 cases
Main Criteria	**Inclusion Criteria:** 1. Males or females aged, 18 to 75 years, inclusive. 2. According to 'The Diagnostic and Therapeutic Criteria for Primary Liver Cancer' (2019 Edition), subject with advanced primary hepatocellular carcinoma, intrahepatic cholangiocarcinoma, combined hepatocellular which is refractory/relapsed after and/or intolerant of standard therapies or for which no standard therapy exists. For the second period of Simon's two-period approach in the Monotherapy extension period, only patients with advanced primary hepatocellular carcinoma were enrolled. 3. There are tumor lesions intrahepatic and / or extrahepatic metastases that can be injected under B ultrasound or CT guidance and meet the volume requirements of the current dose group (the injected lesions are preferably the main tumor burden lesions, see 6.2 Route of administration and method of use for the selection of injectable lesions), and the longest diameter of injectable tumor lesion >1.5cm (or the shortest diameter of lymph node lesions) 4. Eastern Cooperative Oncology Group (ECOG) scores 0 or 1. 5. Life expectancy is at least 3 months. 6. Required organ function: 1) Hematology (no blood transfusion or colony-stimulating factor treatment within 14 days): ANC≥1.5×10^9/L, PLT≥75×10^9/L, Hb≥85g/L; 2) Liver function: TBIL ≤ 1.5 × ULN, ALT ≤ 5 × ULN, AST ≤ 5 × ULN; 3) Child-Pugh A-B level; 4) Renal function: Cr ≤ 1.5 × ULN and creatinine clearance ≥ 45 ml/min (calculated according to Cockcroft-Gault formula); 5) Coagulation function: activated partial thromboplastin time (APTT) ≤1.5 × ULN, prothrombin time (PT) ≤1.5 × ULN, international normalized ratio (INR) ≤1.5 × ULN. 7. HBV-DNA is negative . Subject with positive HBV-DNA must follow 'Guidelines for the prevention and treatment of chronic hepatitis B' (2019 Edition) or clinical routine for treatment. 8. Subjects of childbearing potential (male and female) must agree to use a reliable contraceptive method (hormone or barrier method or abstinence) during the study and for at least 90 days following the last dose; females of childbearing potential must have a negative blood pregnancy test within 7 days of study enrollment. 9. Subjects are required to give informed consent to the study prior to the trial and voluntarily sign a written informed consent form.

Inclusion Criteria:

Subjects are to be excluded from the study if they meet any of the following criteria:

1. Subject in prior anti-tumor therapies such as chemotherapy, radiotherapy, biotherapy, endocrinotherapy, targeted therapy, immunotherapy within 4 weeks of study treatment initiation, including oral fluorouracil and small molecule targeted drugs within 2 weeks before the first dose of IP (Investigational product) or within 5 half-lives of the drug (whichever is longer).
2. Transcatheter arterial chemoembolization(TACE) within 4 weeks of study treatment initiation
3. Participation in clinical trials of any other investigational agents within 4 weeks of study treatment initiation.
4. Major organ surgery (excluding puncture biopsy) or significant trauma within 4 weeks of study treatment initiation.
5. Patients who received systemic treatment with either corticosteroids (>10 mg/ daily prednisone or equivalent) or other immunosuppressive medications within 14 days of study treatment initiation. Patients who have received systemic use of corticosteroids (prednisone > 10 mg/day or equivalent dose of products in the same therapeutic class) or other immunosuppressive agents within 14 days before the first dose of IP, with the exception of the following: use of topical, ocular, intra-articular, intranasal and inhaled corticosteroids; short-term use of corticosteroids for prophylaxis (e.g., prevention of contrast media allergy).
6. Adverse reactions of previous anti-tumor therapy have not recovered to CTCAE 5.0 grading ≤ 1 (except for alopecia and other toxicities that are not considered a safety risk by the investigator).
7. Subjects with central nervous system metastases, or spinal cord metastases..
8. Negative results of herpes simplex virus type I antibody test (both HSV-1 IgG and HSV-1 IgM).
9. Being in a period of recurrent herpes simplex virus infection with corresponding clinical manifestations such as herpes orofacialis, herpetic keratitis, herpetic dermatitis and genital herpes.
10. Other uncontrolled active infections.
11. History of immunodeficiency, including positive HIV antibody testing.
12. History of severe cardiovascular disease:
    1) Ventricular arrhythmia requiring clinical intervention;
    2) QTc interval > 480 ms;
    3) Acute coronary syndrome, congestive heart failure, stroke or other cardiovascular events of grade III and above within 6 months prior to the first dose of study drug;
    4) New York Heart Association (NYHA) classification ≥ Class II or left ventricular

ejection fraction (LVEF) < 50%;

5) Uncontrolled hypertension. (systolic blood pressure≥150 mmHg, or diastolic blood pressure≥100 mmHg after treatment).

13. Active or previous autoimmune diseases that may relapse (such as systemic lupus erythematosus, rheumatoid arthritis, vasculitis, etc.), except clinically stable autoimmune thyroiditis.
14. Previous immunotherapy with an immune-related adverse event (irAE) such as immune-related pneumonia, myocarditis, etc., which, in the judgement of the investigator, may affect the safety of the trial medication.
15. Known alcohol or drug dependence.
16. Subjects with mental disorders or poor compliance.
17. Females during pregnancy or breastfeeding.
18. Subjects who, in the opinion of the Investigator, have other serious systemic diseases or are otherwise unsuitable for participation in this clinical study.

Criteria for treatment discontinuation:

1. Evidence of disease progression and, in the opinion of the investigator, the subject is not deriving clinical benefit from continuing study treatment.
2. Any of the following drug-related adverse events, assessed by the investigator to be possibly, probably, or definitely related to the drug:

(1) Haematological toxicity

-Grade 4 neutropenia lasting > 7 days.

-≥ Grade 3 febrile neutropenia (single measurement of temperature ≥ 38.3 ° C or ≥ 38.0 ° C for 1 hour with concomitant absolute neutrophil count [ANC] < 1000/mm3)

-Grade 4 Thrombocytopenia

-Grade 3 thrombocytopenia with haemorrhage

-Grade 4 anaemia

(2) Non-haematological toxicity

-Grade 4 non-haematological toxicity

-Failure of the following symptoms to resolve to ≤ Grade 2 during treatment 7 days after administration: Grade 3 influenza-like symptoms (e.g., fever, chills, fatigue, body aches or pains, anorexia, dry cough), Grade 3 injection site reactions

-Grade 3 CRS of > 72 hours duration or Grade 4 CRS of any duration

-Any Grade 3 or higher non-haematological toxicity that meets the pre-specified criteria for being at least likely to be caused by the test drug (exclude Grade 3 fever, Grade 3 injection site pain, asymptomatic laboratory test abnormalities at the investigator's judgement).

-Grade 4 immune-related adverse events

	- > Grade 3 colitis or non-infectious pneumonia or myocarditis of any grade (3) Other toxic reactions to the study drug that the investigator determines require permanent discontinuation of the drug. (4) Any Grade 5 AE. The above adverse events were graded according to the NCI CTCAE version 5.0. **Criteria for subject withdrawal:** 1. According to GCP and ICH guidelines, all subjects have the right to withdraw from the study at any time during the trial. 2. Major deviation or protocol violation with impact on tolerability, safety, or pharmacokinetic evaluation of the drug. 3. Withdrawal as determined by the investigator.
Dose and mode of administration	VG161 (recombinant human IL12/15-PDL1B herpes simplex type I oncolytic virus injection (Vero cells)) is supplied at the strengths of 1.0 ml/vial with the appearance of slightly opalescent liquid and the concentration of $\geq 1 \times 10^7$ PFU/ml. Tentatively proposed test sample: Lot No.: 20200702, Titer: 5.93 x 10^7 PFU/ml, Expiry Date: 202200716. VG161 is administered once a day for 1-3 consecutive days of each cycle, with 28 days as a dosing cycle. The method of administration of VG161 is an intratumoral injection, by ultrasound or CT-guided fine-needle puncture of intrahepatic or extrahepatic metastatic lesions. The dose-escalation dose is determined according to the dose group in which the subject is enrolled. The dose administered during the extension period is 3.0 x 10^8 PFU per cycle (1.0 x 10^8 PFU once a day for three consecutive days). The location of the tumour is shown by a diagnostic ultrasound/CT machine. If ultrasound guidance is used, CDFI shows the blood supply within the tumour as well as the large blood vessels surrounding the tumour, and the presence or absence of necrotic areas of the tumour, etc., is shown on the ultrasound image map by injecting 2.4 ml of Sono Vue intravenously.The use of CT instrumentation is carried out in accordance with the regulations of the study centre. Under ultrasound or CT guidance, a PTC needle was used to puncture into the active tumour tissue avoiding blood vessels, and the appropriate volume of VG161 was injected according to the size of the tumour in a manner of injecting while backing out of the needle to allow for uniform distribution of the drug, and after the drug injection was completed, the drug was rinsed with approximately 0.5 ml of normal saline. Refer to the Mylab-Twice ultrasonic diagnostic instrument (Esaote, Italy), the probe is CA541, and the frequency is 2.0-4.0 MHz; The puncture needle is a 18G PTC needle (HAKKO, Japan); the ultrasound contrast agent is Sono Vue (Italy).

Study design

This trial is an open-label phase I clinical trial study for patients with advanced primary liver cancer who have failed standard treatment.

Period 1. Single-agent dose-escalation period

In the single-agent dose-escalation period, 5 dose levels will be set. The starting dose of VG161 is set at 1.0×10^8 PFU, and the preset maximum escalation dose is 5.0×10^8 PFU. During the trial, an intermediate dose may be inserted or the dosing frequency may be adjusted for exploration at the discretion of the investigator based on safety data. The detailed dose group in the dose-escalation part is shown in the table below.

Dose groups	Total dose of VG161 administered per cycle	Predetermined total drug volume [a] (predetermined at drug concentration 5×10^7 PFU/ml)	Mode of Injection[a, b] (Preset at a drug concentration of 5×10^7 PFU/ml)	Number of subjects[c]
1	1.0×10^8PFU	2ml	Single-day	1
2	2.0×10^8PFU	2ml x 2 days	1.0×10^8 PFU daily for 2 days	1
3	3.0×10^8PFU	2ml x 3 days	1.0×10^8 PFU daily for 3 days	3
4	4.0×10^8PFU	2.7ml x 3 days	1.3×10^8 PFU daily for 3 days	3
5	5.0×10^8PFU	3.3ml x 3 days	1.7×10^8 PFU daily for 3 Days	3

a. The specification of the titer is $\geq1\times10^7$ PFU/ml. The actual drug volume to be administered is calculated based on the ex-factory nominal values. The actual total drug volume may be higher or lower than that predetermined at a 5×10^7 PFU/ml drug concentration. It is recommended that the investigator determine the injection volume based on the size of the subject's lesions. Dilution with normal saline is allowed, and the total dose is allowed to be injected into multiple lesions.

b. In the continuous multiple injection dose group, it may be challenging to re-inject the injected lesions, or the subject may have multiple injectable lesions according to the judgment of the investigator; in such case, other lesions can be injected for subsequent treatment, or it can be injected every other day. (See 6.2 Route of administration and method of use for details).

c. Accelerated titration combined with the 3+3 dose-escalation principle is used. Refer to the dose-escalation principle for specific dose-escalation principles and possible number of cases.

The principle of accelerated titration and 3+3 dose escalation is used for dose escalation.

The principle of accelerated titration is used for Dose Group 1 and Dose Group 2. One subject will be enrolled first, and if the subject does not experience Grade 2 or higher toxicity that is definitely or probably related to the test sample (except pyrexia, flu-like symptoms, injection site reactions, injection site pain), the dose will be escalated to the next group, otherwise switch to 3+3. 3+3 dose-escalation approach will be used starting from Dose Group 3.

The MTD was the dose at which $\leq1/6$ subjects in the current dose group would develop a DLT if $\geq33\%$ of subjects in the next higher dose group developed a DLT (i.e., 2/3 or 2/6). If no DLT is observed in the 3 subjects enrolled in the highest dose group, the MTD and the recommended dose for extension groups will be confirmed by the SRC.

During the study, a Safety Review Committee (SRC) consisting of the principal investigator, contract research organisation (CRO) representatives (medical monitor and other relevant personnel) and sponsor representatives, etc., will be established to review the safety data, PK data, and efficacy data generated from the study, to determine the dose level and dosing regimen for each dose increment in the dose-escalation study, to determine the MTDs, and to determine the recommended dosing Dosage. The SRC will also decide whether to add unplanned doses to the trial.

Period 2. Monotherapy extension period

The single-agent dose-escalation phase of this study was completed with DLT observations in all subjects on 30 January 2022, and it was confirmed by the SRC that the maximum safe dose level of 5.0 x 10^8 PFU per cycle had been achieved in this study as no DLT occurred in the trial. In addition, as of 11 October 2021 (the cut-off date for interim analysis data), one patient with intrahepatic cholangiocarcinoma in the 3 x 10^8 PFU per cycle dose group had a best-practice outcome of PR, with a stable disease duration of 3.8 months, and has been treated and observed for 4 cycles; and one subject with hepatocellular carcinoma in the 2 x 10^8 PFU per cycle dose group had a best overall response of SD, with a stable disease duration of 5.1 months, and received 5 cycles of treatment and observation. Based on the results of the safe dose exploration and preliminary efficacy results, the SRC has determined that the recommended dose for the expansion arm is 3.0 x 10^8 PFU per cycle (1.0 x 10^8 PFU once daily for three days). The study will move into the monotherapy expansion phase, which is a single-agent, single-dose and single-arm design, with an indication of advanced primary liver cancer administered at a dose of 1.0 x 10^8 PFU once daily for three consecutive days of intratumoural injections.

Applying Simon's minimal maximum two-Period design (Simon 1989) with a pre-determined Class I error rate of $\alpha = 0.05$ and statistical power $(1-\beta) = 0.85$; assuming a historical control ORR of 11% (referring to the 2019 version of the primary liver cancer diagnostic and treatment guidelines recommending regorafenib as a second-line therapeutic agent, which was derived from a RESORCE study that enrolled 573 patients with Child-Pugh grade A advanced hepatocellular carcinoma who were refractory to sorafenib treatment, (the regorafenib treatment group had an ORR of 11%, a DCR of 65%, a median OS of 10.6 months, and a PFS of 3.1 months based on the mRECIST evaluation), and had an ORR 20% higher than the historical control, at 31%, with VG161. The trial was conducted in two phases according to Simon's two-period design; in the first phase, tumour evaluations were assessed primarily according to RECIST 1.1 and iRECIST criteria, while patients with HCC were evaluated retrospectively using mRECIST. A total of 14 subjects are planned to be enrolled, and after the 14 subjects have completed the first cycle of dosing, the patients' pre-existing data will be comprehensively analysed, and the trial will be considered to be discontinued if there is only 1 or fewer remissions/responses among subjects with pre-existing efficacy assessment based on the 3 evaluation criteria mentioned

above, and if there is no significant benefit of VG161 in other secondary endpoints of efficacy, such as the PFS rate, as compared with the historical control; if, after SRC review, VG161 has clinical benefit in treating patients with hepatocellular carcinoma, such as a higher PFS rate (e.g., 4-month PFS rate) than the historical control data of regorafenib over a certain period of time, or 2 or more PRs, and the available safety and efficacy data show that the benefit of VG161 treatment outweighs the risk, then the trial can be proceeded to the second phase, which is based on the oncological evaluations mainly based on RECIST1.1 and mRECIST criteria, while referring to the iRECIST criteria (determination of disease progression is based on iCPD), and continue to add 16 subjects with advanced primary hepatocellular carcinoma to further investigate the efficacy and safety of VG161 for hepatocellular carcinoma.

Monotherapy Extension Period Study Procedure:

Subjects entered the screening period (-28 days to -1 day) after signing the informed consent form; during the screening period, subjects were required to undergo a series of tests such as performance score (ECOG PS), vital signs, physical examination, blood routine, blood biochemistry, urine routine, herpes simplex virus type I antibody, 12-lead electrocardiogram, imaging (CT/MRI), and so on. Eligible subjects were entered into the trial sequentially according to the inclusion/exclusion criteria.

After enrolment, subjects received VG161 administered at a total dose of 3.0×10^8 PFU per cycle (1.0×10^8 PFU once daily for three consecutive days) according to the recommendations, followed by observation up to day 28 and VG161 efficacy and safety observations according to the protocol. Subjects were treated with the test drug for a cumulative total of no more than 4 cycles of 28 days each, or until disease progression (by iRECIST criteria, iCPD) and in the judgement of the investigator there was no benefit to be derived from continued treatment with the test drug, or intolerable toxicity, or voluntary request for withdrawal, or other reasons for termination of the study. If stability or partial remission of efficacy is achieved after 4 cycles, the decision to continue dosing may be discussed by the SRC (if efficacy is assessed as iUPD, continuation of treatment is still required if the patient is clinically stable and, in the judgement of the investigator, continued treatment may still be of benefit).For details of VG161 dosing, please refer to 6.2 Route of Dosing and Methods of Use.

In consideration of the possibility of pseudoprogression with immunotherapy, if a subject first experiences disease progression (including the appearance of a new lesion), the investigator assesses that the subject is not experiencing rapid tumour progression and the toxicity is tolerable, and the investigator determines that the subject would benefit from continued use of the test drug, the subject will be allowed to continue on the test drug and will be evaluated again for tumour imaging in accordance with iRECIST at least 4 weeks (and not more than 8 weeks) for another tumour imaging evaluation until disease progression (by iRECIST criteria, iCPD) and the investigator determines that there is no

	benefit to be derived from continued treatment with the investigated drug, or intolerable toxicity occurs, or voluntarily requests withdrawal, or other reasons for termination of the study.
Dose-limiting toxicity	A DLT is defined as any of the following toxicities that are related to the IP (including related, probably related, or possibly related) occurring within 28 days of the first cycle of treatment: (1) Haematological toxicity: • Grade 4 neutropenia lasting > 5 days; • ≥ Grade 3 febrile neutropenia (ANC < 1.0 × 10^9/L with a single temperature measurement > 38.3°C or sustained temperature≥ 38.0°C for more than 1 hour); • Grade 4 thrombocytopenia; • Grade 3 thrombocytopenia with bleeding; • Grade 4 anemia; (2) Non-haematological toxicity: • Grade 4 non-haematological toxicity; • Flu-like symptoms (for example pyrexia, chills, asthenia, body aches or discomfort, anorexia, dry cough) of grade 3 and above, injection site reactions of grade 3 and above, and Cytokine release syndrome (CRS) of grade 3 and above that cannot be relieved to grade ≤2 after 7 days of treatment; • Other Grade 3 non-hematological toxicities (except Grade 3 fever and Grade 3 injection site pain), which cannot be relieved to ≤ Grade 2 within 3 days after the best supportive care, except asymptomatic laboratory abnormalities as judged by the investigator; (3) Other toxic reactions that require permanent discontinuation as determined by the investigator. The above adverse events are graded according to the National Cancer Institute Common Terminology Criteria for Adverse Events (NCI CTCAE) version 5.0.
Efficacy evaluation criteria	RECIST 1.1 is a standard method for measuring the response of solid tumours to treatment in clinical studies. In 2008, the American Association for the Study of Liver Diseases (AASLD) proposed the concept of 'surviving tumour' in the design of clinical trials for hepatocellular carcinoma, and revised the RECIST criteria for evaluating the efficacy of immunotherapy, proposing the mRECIST criteria (Modified RECIST Criteria for Evaluation of Efficacy in Solid Tumours). In 2010, the mRECIST evaluation guidelines were publicly published, and after 10 years of clinical trial validation, the mRECIST guidelines were updated in 2020 to further refine the evaluation criteria. The mRECIST criteria used in this trial were the evaluation criteria specified in the 2020 version of the guidelines (see Appendix 7 for details).

	The new standard for judging the efficacy of tumor immunotherapy, iRECIST standard (Immune Response Evaluation Criteria In Solid Tumors), makes up for the shortcoming that RECIST or the modified WHO standard is not fully applicable to anti-tumor immunotherapy. For IUPD on initial assessment by iRECIST criteria (first appearance of a new lesion or at least 20% greater than the nadir of the sum of the diameters of target lesions), the treatment and secondary evaluation may be continued in the absence of acute disease deterioration because shrinkage is likely to begin within 4 weeks after determination of iUPD. iUPD will be considered as iCPD only when there is an increase in tumor burden on two consecutive evaluations (4-8 weeks) and the absolute value of the sum of the measurements increases by at least 5 mm. Efficacy endpoints in the dose-escalation period of this trial, as well as in the first phase of the treatment extension phase, will be evaluated according to the iRECIST and RECIST1.1 criteria, respectively, and retrospectively in patients with HCC using the mRECIST criteria. Efficacy endpoints for Phase 2 of the extension period will be evaluated according to RECIST1.1 and mRECIST criteria, respectively, with reference to iRECIST criteria (iCPD will be used to determine disease progression).
Endpoints	**Period 1. Single-agent dose-escalation period** Primary endpoint 1) MTD/recommended dose; 2) Occurrence and number of DLTs; 3) Occurrence and frequency of AEs, SAEs (according to NCI CTCAE 5.0). Secondary endpoint 1) Pharmacokinetic parameters: Blood concentration of VG161 (copy number of specific VG161 DNA not present in wild-type HSV), PD-L1 blocking peptide and IL12 serum concentrations at different time points after single and multiple doses; 2) Efficacy parameters: objective response rate (ORR), progression-free survival (PFS), duration of response (DOR) and one-year OS rate; 3) Pharmacodynamic and immunology parameters: peripheral blood lymphocyte subsets (CD3+, CD4+, CD8+, CD4+/CD8+ ratio, CD19+, CD16+CD56+ (NK) cells), plasma cytokines (IL-15, IL-6, TNF-a, IFN-γ), peripheral blood T cell immunity and other immune-related indicators (PD-L1, PD-1, CD69, CD8+Ki67high) and serum anti-VG161 antibodies. **Period 2. Monotherapy extension period** Primary endpoint 1) Objective response rate (ORR); Secondary endpoint 1) Progression-free survival (PFS) and PFS rates at specific time points (e.g., 4 months, etc.);

	2) One-year survival rate (OS rate) and overall survival (OS); 3) Duration of response (DOR); 4) Safety evaluation, including adverse events, laboratory parameters and other safety data (electrocardiogram, vital signs, etc.); 5) Pharmacodynamic indicators: peripheral blood lymphocyte subsets (CD3+, CD4+, CD8+, CD4+/CD8+ ratio, CD19+, CD16+CD56+ (NK) cells), plasma cytokines (IL-15, IL-6, TNF-a, IFN-γ), peripheral blood T cell immunity and other immune-related indicators (PD-L1, PD-1, CD69, CD8+Ki67high). The efficacy parameters for the dose-escalation period of this trial, as well as the first period of the monotherapy expansion period of the Simon's two-period design, will be evaluated according to the iRECIST and RECIST1.1 criteria, respectively, and HCC patients will be evaluated retrospectively according to the mRECIST criteria. The efficacy parameters for the second period of the Simon's two-period design to monotherapy expansion will be evaluated according to the RECIST 1.1 and mRECIST criteria, respectively, with reference to the iRECIST criteria (disease progression will be determined by the iCPD).
Statistical methods	The statistical analysis plan (SAP) should be prepared after the protocol finalization and completed before database lock. The SAP will specifically define and describe all planned statistical analyses per the protocol's characteristics. In period 1, the study will focus on descriptive statistics analysis, and in period 2, the study will focus on hypothesis testing and descriptive statistics analysis. 1) Subject baseline parameters Baseline parameters include demographic characteristics and previous treatment history and will be analyzed using descriptive statistics. 2) Safety parameters The parameters include adverse events, vital signs, ECOG score, 12-lead ECG and laboratory tests. Adverse events will be classified and graded according to CTCAE v5.0 and the number of occurrences of adverse events and the number/percentage of subjects will be summarized separately. Drug-related adverse events, Grade 3 or higher adverse events, immune-related adverse events (irAEs), serious adverse events will be summarized separately. Vital signs, ECOG score, 12-lead ECG and laboratory values at each time point will be summarized using descriptive statistics. 3) Pharmacokinetics/ viral shedding The parameters include the distribution of the virus HSV-1 after local administration, the concentration of PD-L1 blocking peptide in the blood, and the serum concentration of IL12. P.K. parameters will be analyzed descriptively. 4) Efficacy parameters The percentage of ORR will be calculated for all subjects and each dose group, and ORR and its confidence interval will be calculated separately for single-agent dose-escalation

	period and monotherapy extension period.. Kaplan-Meier method will be used to estimate the corresponding probability of survival and DOR for all subjects and each dose group, and to estimate median progression-free survival (PFS), survival time (mOS), median DOR and its 95% confidence interval, and curves of PFS, O.S., and DOR will be plotted. 5) Pharmacodynamic parameters and biomarker parameters Preliminary evaluation of changes in immune-related indicators such as blood lymphocyte subsets, cytokines and T cell immunity before and after administration, and preliminary analysis of the correlation with efficacy.

2 List of Abbreviations

AE	Adverse Event
ALB	Albumin
ALT	Alanine Aminotransferase
ALP	Alkaline Phosphatase
ANC	Neutrophil Count
APTT	Partial Thromboplastin Time
AST	Aspartate Aminotransferase
BUN	Urea Nitrogen
Ca	Blood Calcium
Cl	Blood Chlorine
Cr	Creatinine
CRF	Case Report Form
CRS	Cytokine Release Syndrome
DBil	Bilirubin Direct
DCR	Disease Control Rate
DLT	Dose Limiting Toxicity
DNA	Deoxyribonucleic Acid
FAS	Full Analysis Set
GCP	Clinical Trial Management Practices
GGT	Gamma-Glutamyl Transpeptidase
GLU	Fasting Plasma Glucose
H	Hours
Hb	Haemoglobin
HCC	Hepatocellular carcinoma
HIV	Human Immunodeficiency Virus
HSV-1	Herpes Simplex Virus, Type I
INR	International Normalized Ratio
ICC	Intrahepatic cholangiocarcinoma
iCPD	Disease Progression according to iRECIST
irAE	Immune-Related Adverse Events
iRECIST	Immune Response Evaluation Criteria in Solid Tumors
ITT	Intention to Treat
K	Blood Potassium
LVEF	Left Ventricular Ejection Fraction
Mg	Blood Magnesium
Min	Minutes

MTD	Maximum Tolerance Dose
Na	Blood Sodium
NOAEL	No-Observed-Adverse-Effect
NYHA	New York Heart Association
ORR	Objective Response Rate
P	Blood Phosphorus
PFS	Progression-Free Survival
PFU	Plaque-Forming Unit
PLT	Platelets
PK	Pharmacokinetics
PT	Prothrombin time
RBC	Red Blood Cell Count
RECIST1.1	Response Evaluation Criteria 1.1 of Solid Tumors
RNA	Ribonucleic Acid
SAE	Serious Adverse Event
SOP	Standard Operating Procedures
SRC	Safety Review Committee
SS	Safety Set
TACE	Transcatheter arterial chemoembolization
TBil	Bilirubin Total
ULN	Upper Limit Of Normal
UREA	Urea
WBC	Leukocyte Count

Main Body of the Study Protocol

1 Introduction

Cancer is a disease that seriously endangers human health and is characterized by abnormal cell proliferation and metastasis. Cancer is the second leading cause of death in China behind cardio- and cerebrovascular diseases. According to the national cancer statistics (2019) released by the National Cancer Center, there were about 3.929 million cases of malignant tumors in China in 2015, with 2.338 million deaths. The incidence rates of malignant tumors increase an average of 3.9% per year; the mortality rate increases an average of 2.5% per year. With the growing morbidity and mortality, cancer is becoming the leading cause of death and a vital public health issue in China.

Primary liver cancer is currently the fourth most common malignant tumor in China, which seriously threatens the lives and health of people in China. Primary liver cancer mainly includes three different pathological types: hepatocellular carcinoma (HCC), intrahepatic cholangiocarcinoma (ICC) and HCC-ICC mixed type, of which HCC accounts for 85% to 90%. HCC cases in China account for 55% of global cases, It is the third most fatal tumor in China. Due to the insidious onset of HCC, up to 80% of patients have unresectable or metastatic advanced disease at the time of initial diagnosis. In general, patients with advanced HCC have rapid disease progression and poor prognosis. The 5-year survival rate is only 12.1%. The median survival time of advanced HCC in China is less than 1 year. HCC treatment methods include liver resection, local ablation therapy, liver transplantation, transcatheter arterial chemoembolization (TACE) and systemic chemotherapy, targeted therapy and immunotherapy; however, after treatment, it is prone to recurrence and metastasis, and the long-term efficacy is poor. Improving survival has always been an important treatment goal for advanced HCC.

1.1 Rationale

Oncolytic viruses can specifically replicate in tumors, breaking tumor cells apart without damaging normal cells. These viruses may enter normal cells, but they cannot replicate. According to their genome, RNA virus and DNA virus are the two main categories. In 2005, the world's first oncolytic adenovirus (Shanghai Sunway Biotech Co., Ltd., Ankerui) for the treatment of advanced head and neck tumor was approved by NMPA (formerly CFDA); in 2015, a GM-SCF expressing herpes simplex oncolytic virus (T-Vec) for the treatment of relapsed and refractory advanced melanoma produced by Amgen was approved by the US FDA.

The mechanism of tumor-specific replication of oncolytic viruses:

Many factors contribute to the feature that the oncolytic virus replicates explicitly in tumor cells without spreading in normal cells and causing toxicity. The main reason is that when normal cells turn into tumor cells, biological changes occur, resulting in generally lower intracellular antiviral activity in tumor cells than in normal cells. The reasons include: 1) The most common reason is that the IFN signaling pathway in tumor cells that inhibits virus growth becomes defective. There

seems to be a correlation between the defect and oncogenic alterations, and many viruses can replicate in cells. 2) High expression of some tumor genes such as RAS directly facilitate virus replication (recent reports show that high RAS also inhibits the IFN signaling pathway). 3) Highly expressed DNA repair genes contribute to virus replication as well. 4) Normal cells would initiate apoptosis mechanism after infection. The infected cells would be removed quickly, inhibiting the spread of the virus. This is an essential mechanism for cells to resist viral infection. However, for tumor cells with apoptosis pathway defects, apoptosis cannot be triggered rapidly for virus-carrying cells, resulting in the virus replicating effectively in the cell. 5) The high expression of AKT in tumor cells is inevitably related to the anti-apoptotic mechanism. Studies have found that, for some viruses, the high expression of AKT directly contributes to the replication. 6) The promoter regions of key genes carried by many viruses contain the NFkB binding domains and these key genes can be highly expressed in cells with high expression of NFkB. Tumor cells, especially tumor stem cells, mostly have NKkB activity, which facilitates the growth of viruses. Notably, the above highly expressed genes are the resistance mechanism of tumor cells; therefore, oncolytic viruses are sometimes more effective for drug-resistant tumors.

The anti-tumor mechanism of oncolytic virus:

The anti-tumor oncolytic virus acts through multiple mechanisms, including but not limited to the following aspects: First, lytic replication of the oncolytic virus. The multiple mechanisms, including specific receptor binding, non-specific cell membrane fusion, phagocytosis, macropinocytosis, etc., contribute to the virus entering into tumor cells. After invading, the structural proteins carried by the virus activate the replication machinery immediately. Simultaneously, many viruses carry or express viral proteins that prevent the transport of the host cell's mRNA from the nucleus to the rest of the cell, thereby hijacking the cell's protein synthesis machinery for the virus. This process eventually causes the lysis of host tumor cells. Second, the oncolytic virus destroys the vascular structure of the tumor. In the tumor infected by the oncolytic virus, the virus can also enter into the vascular endothelial cells, thereby causing the apoptosis of endothelial cells, disrupting the vascular structure and affecting the tumor's blood supply. Third, immune response. This is one of the most important mechanisms by which the oncolytic virus kills tumors and this area has been most intensely studied. The immune-related tumor cell-killing mechanism caused by oncolytic viruses can be summarized as follows: 1) Change the immune microenvironment of tumor cells. The tumor microenvironment plays a decisive role in tumor growth, metastasis and drug resistance. Tumor cells cannot live without the support of other non-tumor cells within the tumor, including intratumoral stromal cells and other cells associated with the tumor, such as immune cells. The initial manifestation of virus-infected tumors is the microenvironment change. This is because the infected cells release large amounts of different cytokines, which recruit peripheral circulating immune cells to infiltrate the tumor. These cells are primarily non-specific immune cells, such as macrophages, neutrophils, N.K. cells and D.C. cells. Evidence has shown that some original tumor-associated macrophages alter from M2 type (nutrient type) to M1 (kill type) due to virus infection. Therefore, the tumor microenvironment

change can improve the tumor-killing activity of the original immune cells that coexist with the tumor. 2) Immunogenic cell death (ICD). It is known that when tumor cells are subjected to radiotherapy or some chemotherapeutic agents, a series of cell responses such as E.R. stress can be triggered. These cell responses express some factors on the cell surface, which is known as damage-associated molecular patterns (DAMPs). Cells with DAMPs would be killed by attracting immune cells, which is the immunogenic cell death (ICD). Many virus-infected cells also have DAMPs. As described in 1) above, many immune cells are recruited into the infected tumor, allowing an abundance of immune cells to enter the tumor that initially lacks immune cell infiltration and kill the cells with DAMPs. Therefore, in an infected tumor, even if the virus does not directly lyse the tumor cells for various reasons, the cells will be eliminated because of ICD. Simultaneously, since tumor cells (including uninfected cells) have a relatively low expression of MHC on the cell surface, these uninfected tumor cells would be killed as foreign invaders after a large number of N.K. cells infiltrated the tumor. Therefore, intratumoral infiltration of non-specific immune cells kills infected and uninfected tumor cells. 3) T-cell-mediated specific tumor killing. Viral infection of tumors leads to a large quantity of intratumoral infiltration of macrophages and D.C. cells, all of which are antigen-presenting cells (APCs). As they phagocytize infected tumor cells and their tumor cell debris formed due to viral oncolysis, they will present tumor cell antigens to T cells that are recruited into the tumor together. In the tumor microenvironment altered by the virus, these T cells have high activity and CD8+ T killer cells activated by antigen presentation can specifically recognize the tumor cells expressing tumor antigens and kill specifically. More importantly, during this process, CD8+ cells also produce an immune memory of such tumor antigens, thereby forming a systemic, persistent anti-tumor immunity, allowing the immune system to recognize and kill distal metastasis other than the infected tumor in situ and preventing recurrence. In animal models and clinical cases of T-VEC, tumor ablation by oncolytic virus inoculation is often followed by the disappearance of the same tumors or metastases distally. It is suggested that immunity plays a significant role in the tumor-killing mechanism of oncolytic viruses.

1.1.1 Rationale of VG161

VG161 (recombinant human IL12/15-PDL1B herpes simplex type I oncolytic virus injection (Vero cells)) is a type I herpes simplex oncolytic virus expressing IL12, IL15/15RA (IL15 and IL15 receptor α subunits) and PD-L1 blocking peptide (PDL1B) independently developed by CNBG Virogin Biotech (Shanghai) Ltd. Its genome and genetic modification are shown in the figure below (Figure 1):

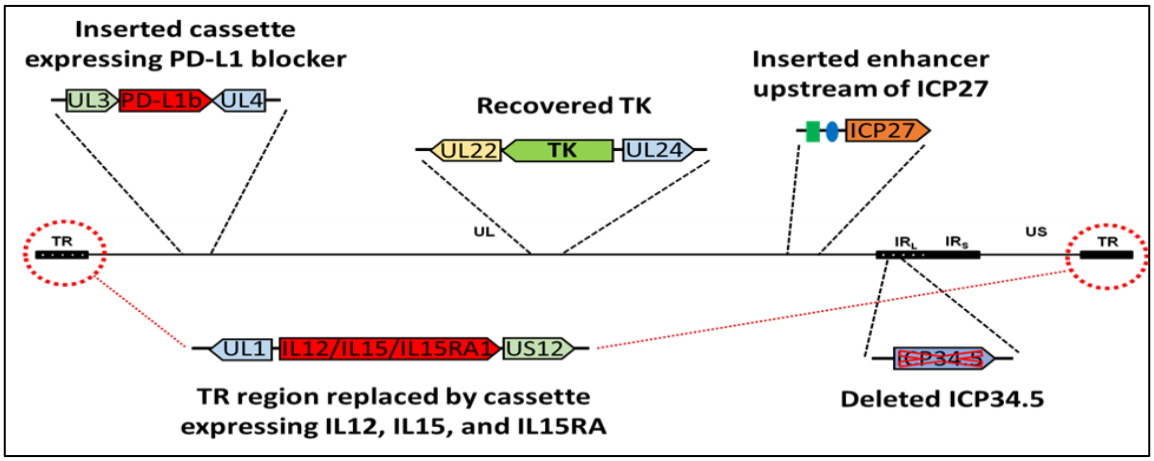


Figure 1: Structure of VG161

To remove the neurological effects of the virus and enhance selective replication of the tumor, two copies of the RL1 gene encoding ICP34.5 were deleted. The promoter region of the very early gene ICP27, which was necessary for virus replication, was inserted into the NF-kB/Oct4 response element to increase the virus's specific replication in tumor cells. An expression cassette of secreted PD-L1 blocking peptide (TF+Fc) fused to human Fc was inserted between the two genes UL3 and UL4. This expression cassette was controlled by the EF1a promoter and bidirectional SV40 polyadenine nucleoside (poly A). The terminal repeat region (T.R.) was deleted to generate a transgene insertion space of about 15 kb, but the original ICP47 promoter (part of T.R.) and ICP47 coding region of the virus were retained. The expression of ICP47 was beneficial to delay the immune clearance of virus-infected cells, improving the persistence of expression and expanding the time window for armed gene delivery. An expression cassette expressing secreted IL12, IL15 and IL15 receptor alpha subunits was inserted into the position previously occupied by the T.R. region. This expression cassette was controlled by the CMV promoter and bGH polyadenine nucleoside.

1.1.2 Innovative features of VG161

First, VG161 deletes ICP34.5 (two copies) in the herpes simplex virus type I genome to ensure the safety of the virus backbone; second, IL12, IL15/15RA and PDL1B are innovatively inserted to obtain synergistic anti-tumor immunostimulatory activity. Previous pharmacological studies have shown that in addition to the direct oncolytic effect, the IL12, IL15/15RA and PDL1B carried by VG161 synergistically have anti-tumor immunostimulatory effects. In the in vivo pharmacodynamic model, local intratumoral injection of VG161 eliminates the injected tumor and has systemic anti-tumor effects and forms immune memory by inducing the anti-tumor immunity. Biodistribution and toxicology studies show that VG161 is safe.

The innovative features of VG161 are as follows: 1) While deleting ICP34.5 to attenuate, VG161 retains the original T.K. gene of HSV-1, making the virus highly sensitive to the antiviral drug acyclovir and allowing rapid in vivo inactivation of the virus when necessary. 2) Based on the ICP34.5-deficient virus backbone described above, an enhancer responsive to NFkB/Oct4 is added to the promoter region of the immediate early gene ICP27 of the virus in the VG161. Since the

two transcription factors, NFkB and Oct4, are highly expressed in tumor cells and tumor stem cells, the enhancer can enhance the transcriptional activity of ICP27 in tumor cells after being inserted into the promoter region of ICP27. 3) One difference between VG161 and T-Vec is that VG161 retains the original gene ICP47 of the virus, which mainly blocks the transport of MHC proteins to the cell surface, preventing the virus from being prematurely eliminated by T-cells that recognize HSV antigens. Since VG161 retains ICP47, the virus can "hide" in the infected cell for a longer time, persistently releasing the exogenous immunostimulatory genes carried by VG161 and resulting in a higher concentration of immunostimulatory factors in the tumor. 4) The significant difference between VG161 and other HSV oncolytic viruses is that VG161 carries 4 immunomodulatory genes: IL12, IL15, IL15RA and PDL1B (TF-Fc). All of the aforementioned exogenous gene products are efficiently released outside the infected cell. These proteins significantly alter the immune microenvironment within the tumor, creating an anti-tumor immune environment contributed by the virus-induced antiviral immune response. VG161 is the world's first oncolytic virus expressing multiple genes with synergistic anti-tumor immunostimulatory effects.

Synergistic immunostimulatory effect of IL12, IL15/IL15RA and PDL1B:

In vitro studies have shown that tumor cells (293 cells, U87 and MDA-MB-231) infected with oncolytic viruses expressing both IL12 and IL15/IL15RA produced significantly higher levels of IFNγ and TNFα after co-culture with human PBMCs than those infected with oncolytic viruses expressing IL12 or IL15/IL15RA alone after co-culture with human PBMCs. Cytotoxicity tests also showed that oncolytic viruses expressing both IL12 and IL15/IL15RA are highly cytotoxic.

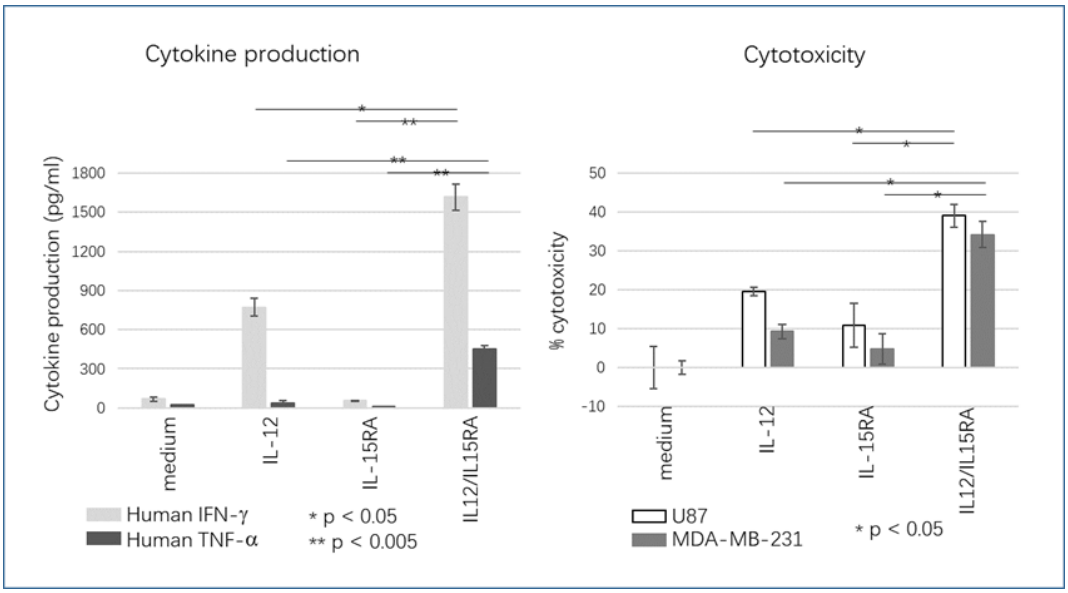


Figure 2: Synergistic immunostimulatory and cytotoxic effects

Note: Left panel: 293 cells were infected with vectors expressing IL12, IL15/IL15RA alone, or co-expressing IL12 and IL15/IL15RA and then co-cultured with PBMCs to observe the expression of INF-γ and TNF-α in PBMC cells. The results showed that the co-expression of IL12 and IL15/IL15RA synergistically stimulates PBMC cells to release INF-γ and TNF-α to the greatest degree. This difference was significant compared with the control group, IL12 alone or IL15/IL15RA alone group. Right panel: U87 (human glioma) and MDA-MB-231 (human breast cancer) cells were infected with vectors expressing IL12, IL15/IL15RA alone or co-

expressing IL12 and IL15/IL15RA and then co-cultured with PBMC cells to observe the cytotoxic effect of PBMC cells on U87 and MDA-MB-231 cells. The results showed that IL12 and IL15/IL15RA co-expression vectors stimulate PBMC cells to produce cytotoxicity to the greatest degree. This difference was significant compared with the control group, IL12 alone or IL15/IL15RA alone group.

VG161 stimulated PBMC to produce the highest level of IFNγ compared with VG160 (virus backbone), VG160-PDL1b (expressing PDL1B alone), VG160-15RA1 (expressing IL15RA alone), VG160-RA1-PDL1b (expressing IL15RA+PDL1B alone) and VG160-h1215 (expressing IL12+IL15RA alone).

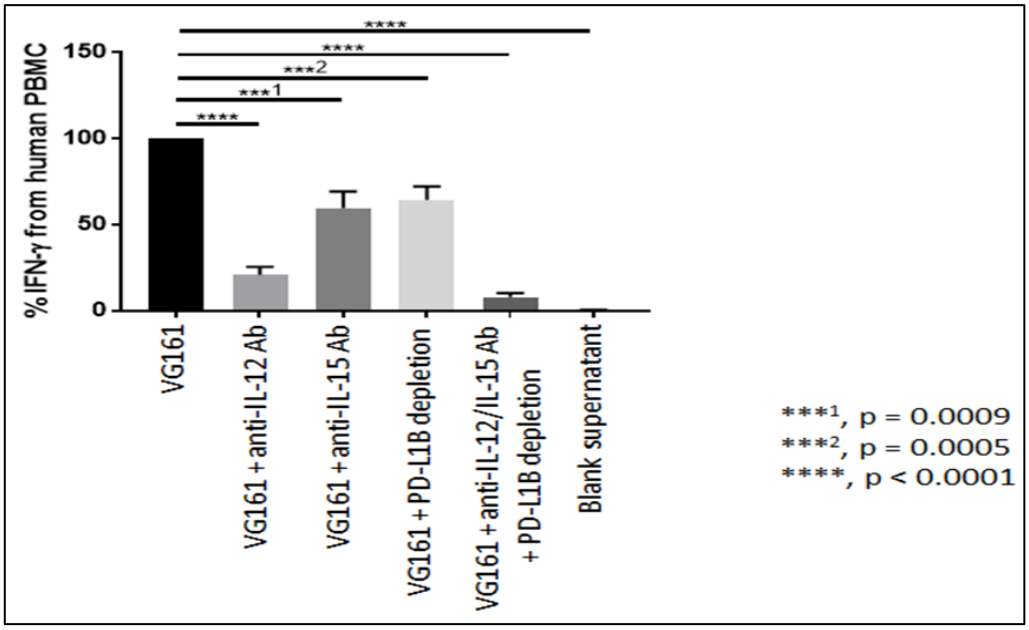


Figure 3: Synergistic immunostimulatory effect of three exogenous genes

Note: Tumor cell-infected VG161 co-cultured with PBMCs in the presence of IL12 neutralizing antibody (VG161+anti-IL-12Ab), IL15 neutralizing antibody (VG161+anti-IL-15Ab), VG161+PDL1B depletion removed using an affinity chromatography column, respectively, or treated with the above three together (VG161+anti-IL-12/IL-15Ab+PDL1B depletion). The results showed that blocking the effect of an exogenous gene with neutralizing antibodies or blocking all three exogenous genes could reduce INF-γ expression in PBMC cells significantly. The mean results using 4 different humanized PBMCs are shown.

1.2 Pre-clinical pharmacodynamic studies

In vitro test

The in vitro tests were conducted systematically to study the inhibition of VG161 on a variety of tumor cell lines. The inhibition of cell proliferation was studied on 11 tumor cell lines, including lung cancer H23s, A549, H1975, H460, liver cancer HepG2, Hep3B, pancreatic cancer BxPC3, gastric cancer AGS, prostate cancer PC9, breast cancer MCF-7 and colon cancer LS174T, of which 7 tumor cell lines (H460, H1975, HepG2, H23s, BxPC3, AGS, A549) showed IC_{50} of less than 1 MOI. Besides, the proliferation characteristics of VG161 on tumor cell line FaDu and normal cell line MRC-5 were also studied. The results showed that the replication and proliferation of VG161 in tumor cells at MOI=0.01 was 384 times higher than that in normal cells, indicating that VG161 has specific proliferation characteristics in tumor cell lines.

In vitro infection of tumor cells with VG161 resulted in detectable expression of exogenous gene

products, which stimulates the anti-tumor immune function of PBMC. The IL12, IL15/15RA and PDL1B bound to receptors on target cells or bound to PD-L1 protein in vitro and further triggered receptor-specific immune responses. VG161 exogenous genes synergistically stimulated anti-tumor immune function in vitro and stimulated the body's immune response.

In vitro pharmacodynamic tests have proved that VG161 has a broad-spectrum anti-tumor effect.

In vivo test

In vivo pharmacodynamic studies have shown that VG161 shows a strong oncolytic effect and stimulates the body's immune response to form specific anti-tumor immunity. It achieves the synergy of oncolytic activity and immune stimulation, and the synergy between the various immunostimulatory factors expressed in the tumor carried by the virus; and leads the antiviral immune response to the anti-tumor immune response to produce a synergistic anti-tumor effect.

In the subcutaneous transplantation tumor model of human liver tumor cells, VG161 showed a solid oncolytic effect. After multiple continuous injections and multiple administration courses, the tumor volume could be significantly reduced, and some mouse tumors disappeared completely.

In a tumor-bearing mouse model with normal immunity, intratumoral injection of mVG161 expressing murine genes made some CT26 mouse-derived colon cancer subcutaneously transplanted tumors completely disappear; subsequent resistance occurs against the second subcutaneous transplantation of CT26 tumor cells; and the second inoculation could not form tumors. These prove that local intratumoral injection of VG161 can activate the body's adoptive immune system, and induce the body's systemic and specific anti-tumor immune memory. VG161 could not only still significantly inhibit tumor growth in HSV antibody-positive mice, but also had a faster anti-tumor effect than in HSV antibody-negative mice. The antibody-positive group had a smaller tumor volume than the antibody-negative group, with a higher proportion of tumors that disappear entirely, suggesting that the HSV antibody produced by mice might enhance the anti-tumor effect of VG161. It is demonstrated that anti-HSV-1 antibodies do not affect the efficacy of repeated administration of VG161.

In vivo trials studied the inhibitory effect of VG161 on multiple tumor cell lines transplanted tumor models. Among the models, the human liver tumor cell subcutaneous transplantation tumor models include: nude mouse human liver cancer (Hep3B) model and humanized immune reconstruction mouse subcutaneous transplantation tumor model of intrahepatic cholangiocarcinoma. Besides, the models include colorectal cancer (CT26) and reticulocyte sarcoma (A20) models in immunocompetent mice, human gastric cancer (SGC-7901) and human glioma (U87) models. The trials mainly observed the effect of VG161 on tumor volume, relative tumor proliferation rate (T/C%), relative tumor inhibition rate (TGI%), animal weight, animal survival time, anti-tumor immune stimulation, etc.

In the nude mouse model of human liver cancer (Hep3B), the average volume of subcutaneous tumors reached about 100 mm^3. VG161 was injected intratumorally at low, medium and high doses (2.5×10^5, 1.2×10^6 and 6.2×10^6 PFU/ mouse/day, once a day for 5 consecutive days) and showed significant anti-tumor activity in all groups. The T/C% was 22.47%, 15.31% and 24.81%,

respectively, reaching the effective criteria and the efficacy was superior to the positive control drug oxaliplatin.

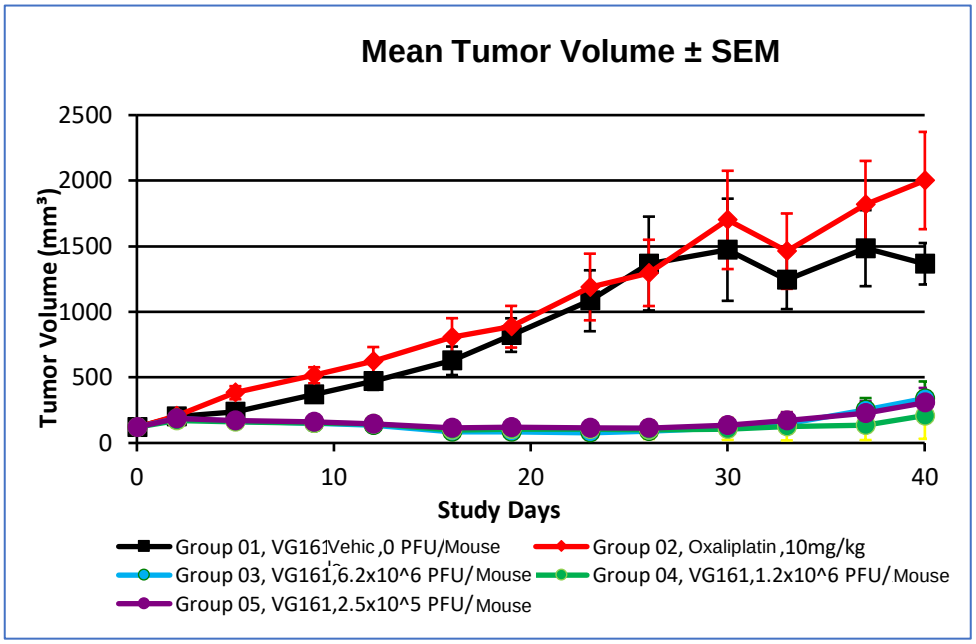


Figure 4. Tumor volume change curve on Hep3B mouse model of liver cancer in each group treated with VG161

The in vivo efficacy of VG161 was tested on a humanized immune reconstitution mouse (HuNPI) subcutaneous transplantation tumor model taken from a clinical patient liver cancer tissue (intrahepatic cholangiocarcinoma) (PDX). When the tumor volume reached 80-180 mm^3, VG161 was injected intratumorally at low and high doses (3.1×10^5 and 7.75×10^6 PFU/mouse/day, once a day for 5 consecutive days, 28 days for 1 cycle, for a total of 3 cycles). The experimental results showed that VG161 had a significant inhibitory effect on tumor growth ($p<0.05$), with T/C% of 42.01% and 27.23% in the low and high dose groups. It is 42.72% in the positive control group gemcitabine & oxaliplatin). The initial effective dose was 3.1×10^5 PFU/mouse. The high-dose group had better efficacy, significantly prolonged overall survival and better safety compared with the combination chemotherapy group. The above findings suggest that VG161 exhibits significant oncolytic capability and exerts a stimulatory effect on the body's immune response, thus resulting in specific anti-tumor immunity leading to continuous reduction in tumor volume.

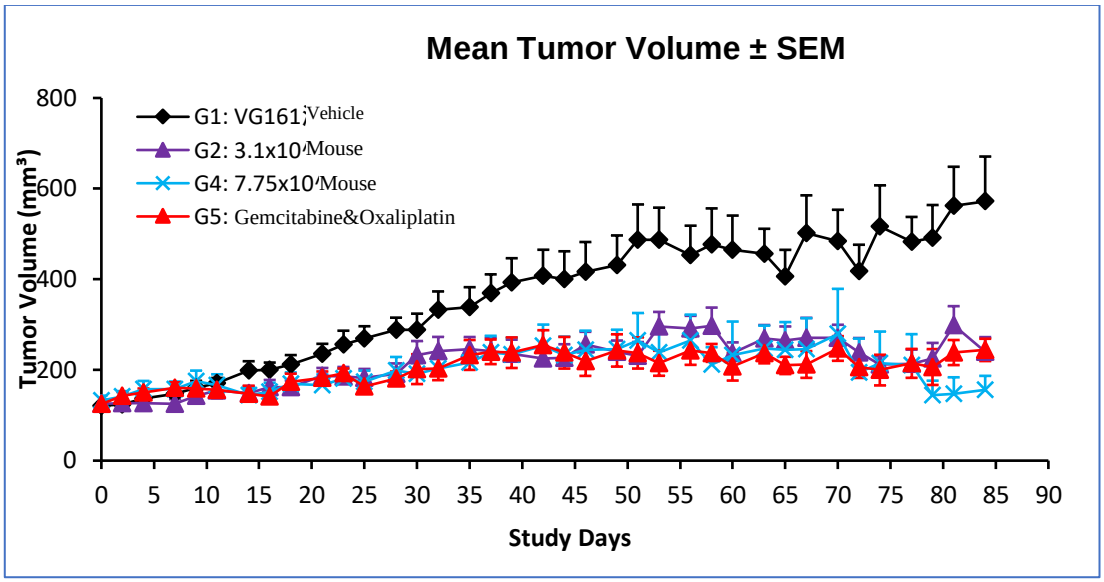


Figure 5. Tumor volume change curve of VG161 in human liver cancer PDX model of humanized immune reconstituted mice of each group

VG161 also showed a strong anti-tumor effect in tumor models of other tumor types. It can be oncolytic and activate systemic anti-tumor immune responses to achieve systemic anti-tumor effects.

In the nude mouse model of gastric cancer (SGC-7901), the average volume of subcutaneous tumors reached about 100 mm^3. VG161 was injected intratumorally at low, medium and high doses (9.08×10^5, 4.54×10^6, and 9.08×10^6 PFU/mouse/day, once every other day for 4 consecutive times) and showed significant anti-tumor activity in all groups with some dose-dependence. The T/C% of the high-dose group was less than 40%, which met the effective criteria and the efficacy was superior to the positive control drug 5-Fu.

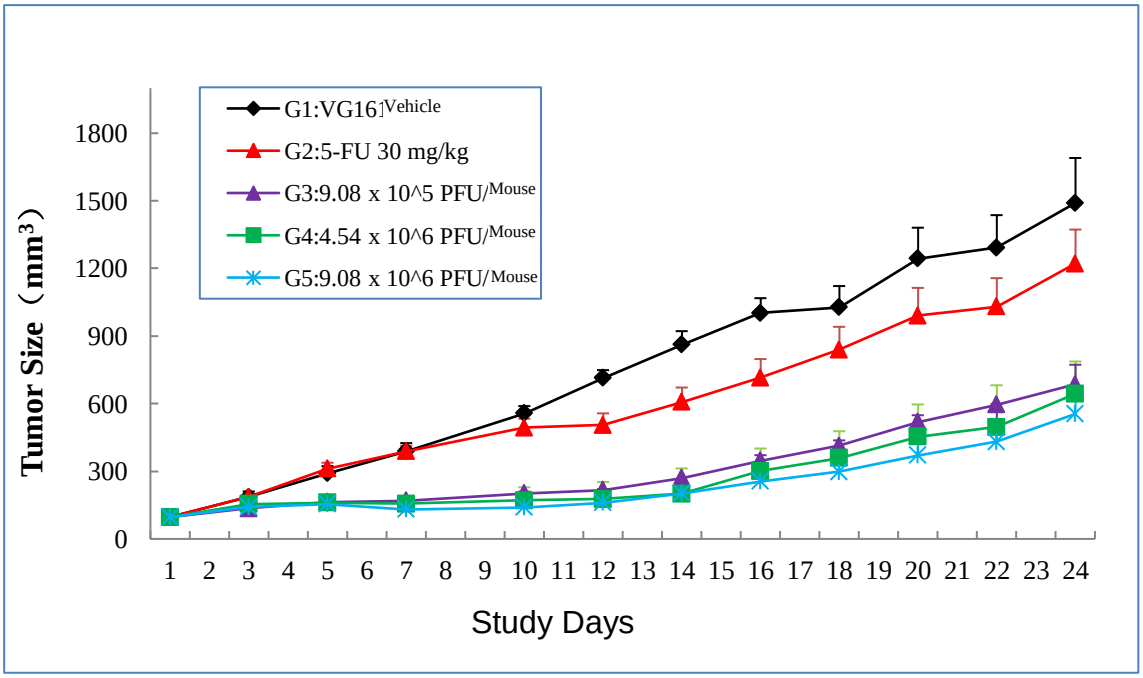


Figure 6. Tumor volume change curves of VG161 on SGC-7901 mouse model in each group

In addition, VG161 can significantly inhibit tumor growth in HSV antibody-positive mice and induce the anti-tumor effect faster than HSV antibody-negative mice. The antibody-positive group showed a smaller tumor volume and higher proportion of complete recession of tumor than the antibody-negative group, suggesting that the HSV antibody produced by mice might enhance the anti-tumor effect of VG161 after HSV pre-immunization. The results suggested that anti-HSV-1 antibodies did not affect the efficacy of repeated doses of VG161.

The in vivo efficacy of different doses of mVG161 expressing murine genes were tested on the subcutaneously transplanted mice model of the CT26 mouse-derived colorectal cancer in immunocompetent mice (Balb/c). The test results showed that intratumoral injection of mVG161 had anti-tumor activity at high, medium and low doses (5×10^7, 5×10^6 and 5×10^5 PFU/mouse/day, once a day for 5 consecutive days) and had no significant effect on the weight of mice. The anti-tumor effect of the high-dose mVG161 group had the best efficacy with 80% of complete tumor disappearance. The medium- and the low-dose group also inhibited tumor growth in mice, with 50% and 20% of complete tumor disappearance, respectively.

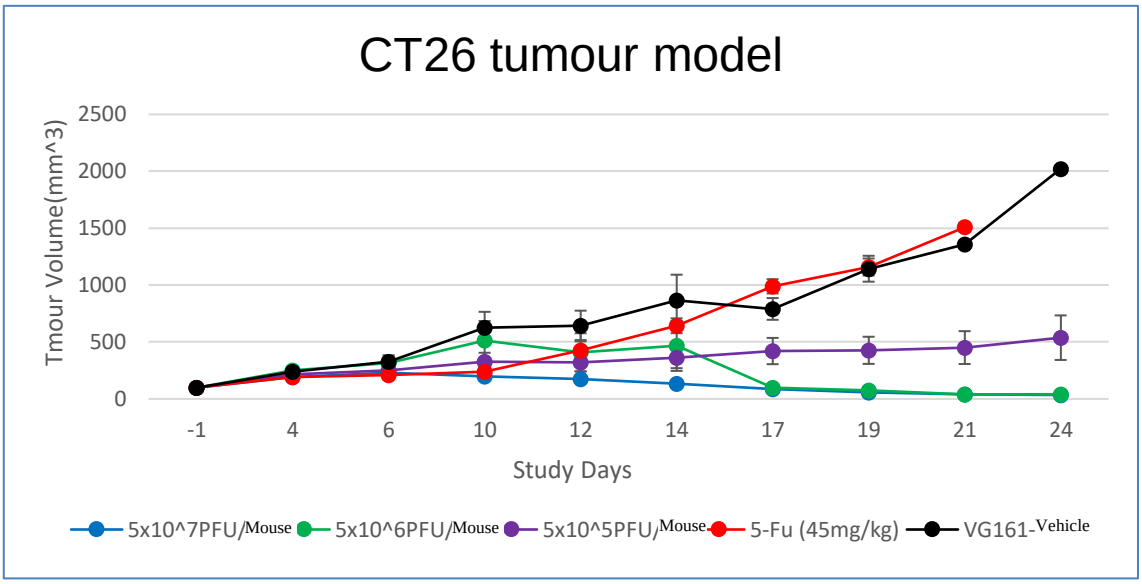


Figure 7. Tumor volume change curve on CT26 mouse model in each group treated with mVG161

On the subcutaneously transplanted mice model of CT26 colorectal cancer in immunocompetent mice (Balb/c), the effect of mVG161 expressing murine gene on inducing anti-tumor immune memory in mice and the resistance to repeated CT26 tumor cell transplantation were observed. The test results showed that intratumoral injection of mVG161 led to the complete disappearance of CT26 colorectal cancer transplanted tumors with no recurrence within about 78 days of the disappearance (after day 15 of the test), indicating that VG161 had significant anti-tumor activity. The CT26 was injected into the previously mVG161-treated tumor-bearing mice again 78 days after the complete tumor disappearance (i.e., day 92 of the test), no tumor developed; the CT26 was injected into the mice of similar age with no mVG161 treatment, tumors developed and grew naturally. The results showed that VG161 had significant anti-tumor activity, stimulated the body's

immune system and induced its anti-tumor immune memory. It was suggested that VG161 prevented or stopped tumor recurrence and metastasis.

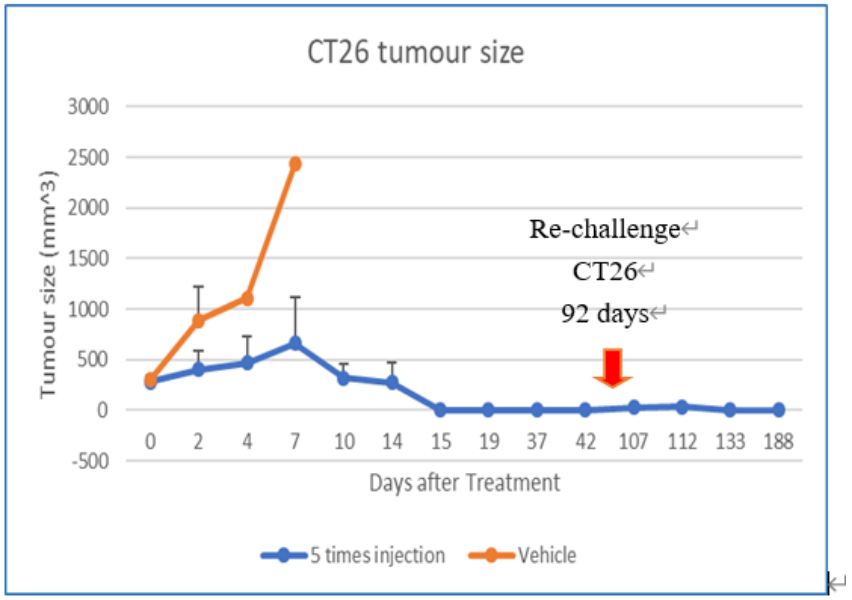


Figure 8. Intratumoral injection of mVG161 led to complete response of CT26 tumors and induced long-term immune memory

The in vivo tumor inhibitory effect of different doses of mVG161 expressing murine genes was tested on the unilateral transplanted mice model of A20 reticulocyte sarcoma in immunocompetent mice (Balb/c). The results showed that intratumoral injection of mVG161 at high, medium and low doses (1×10^7, 5×10^6, and 1×10^6 PFU/mouse/day, once a day for 5 consecutive days) significantly inhibited the A20 tumor growth in mice compared with the vehicle control. By the end of the pharmacodynamic study, the tumor volume of the mice in all three dose groups was below 600 mm^3, while the vehicle control group was above 2500 mm^3. Mice in all treatment groups survived and showed significant anti-tumor activity.

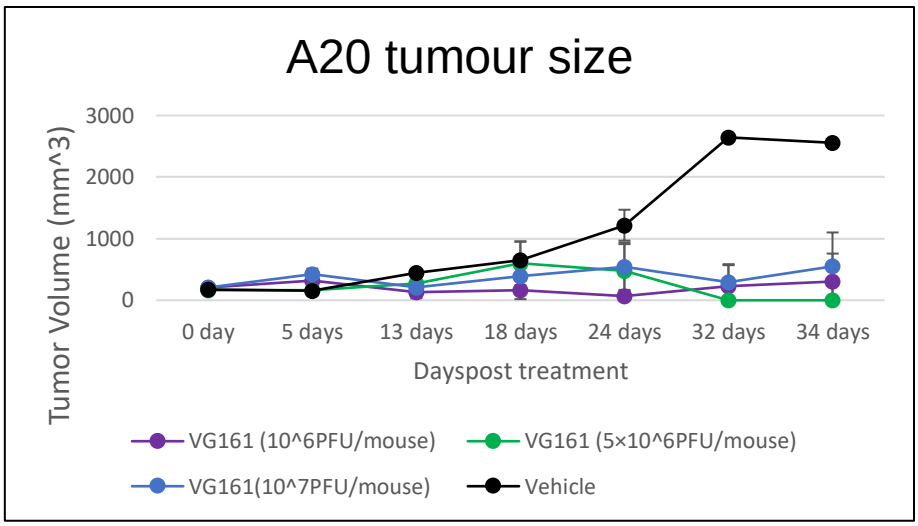


Figure 9. Tumor volume change curve on A20 unilateral mouse tumor model in each group treated with mVG161

The systemic immune system regulation and the inhibitory effect on distal tumors of mVG161 expressing murine genes were tested on the bilateral transplanted mice model of A20 reticulocyte sarcoma in immunocompetent mice (Balb/c). The A20 bilateral subcutaneous tumor model was created. One side of the tumor was injected intratumorally when the average volume of subcutaneous tumors on both sides reached about 100 mm^3 to observe the systemic anti-tumor immune regulation of VG161. The test results showed that VG160 (the backbone virus of VG161, with no IL12, IL15/15RA and PDL1B exogenous genes) and the vehicle control could not control the tumor growth in mice. In the mVG161 group, the tumor volume on the injected side shrank starting on day 21 and eventually disappeared entirely. The tumor volume on the non-injected side also tended to decrease after day 21. By day 52, the tumors on both sides of the mice had disappeared entirely. VG161 effectively inhibited tumors on the injected side and had a solid immunosuppressive effect on tumors on the non-injected side. With the synergistic effect of the inserted immunomodulatory factors, VG161 could activate the systemic anti-tumor immune response, achieving a systemic anti-tumor effect. The results suggested that local intratumoral injection of VG161 had anti-tumor effects on non-injectable small or deep lesions at the distal end.

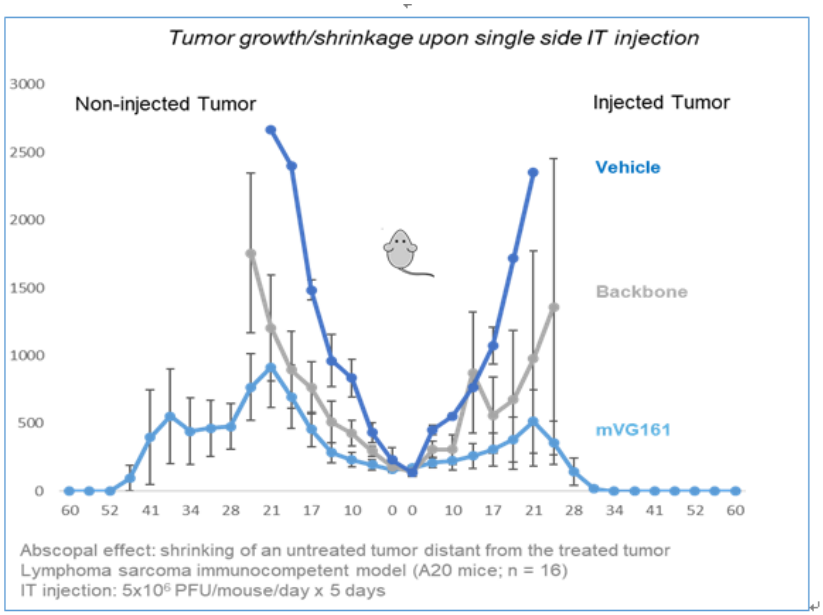


Figure 10. Tumor volume change curve on A20 bilateral tumors in each group following unilateral intratumoral injection of mVG161 virus

1.3 Preclinical safety

Safety pharmacology studies were conducted in rats and monkeys. Single-dose toxicity studies and repeat-dose toxicity studies (up to 4 cycles of treatment) were conducted in rats and cynomolgus monkeys, and no significant toxic reactions were observed except for local injection reactions. (Please see the Investigator's Brochure for details of pre-clinical safety test data)

Single-dose toxicity study

After a single intramuscular injection of VG161, Sprague-Dawley rats were observed for acute toxicity that might occur within 14 days. Twenty rats (n=10/gender) were assigned into 2 groups (n=5/ gender/group) according to weight to receive vehicle control (vehicle-VG161) and VG161 at a dose of 4.46×10^8 PFU/mouse via intramuscular injection. The dose administered was about

95.3 times the lowest effective dose in rats. A single intramuscular injection of VG161 at a 4.46×108 PFU/mouse dose was administered to Sprague-Dawley rats. No treatment-emergent abnormal changes were observed in clinical indicators, body and food intake, and the maximum tolerated dose (MTD) was greater than or equal to 4.46×10^8 PFU/mouse.

Similarly, cynomolgus monkeys were observed for acute toxicity that might occur within 14 days after a single intramuscular injection of VG161. All animals were assigned into four groups (n=1/gender/group) by weight to the receive vehicle control(vehicle-VG161) and VG161 at doses of 2.97×10^7, 2.97×10^8, and 2.97×10^9 PFU/mouse, respectively. The doses administered were approximately 1, 10 and 100 times the lowest effective dose in monkeys. Under the conditions of this experiment, a single intramuscular injection of VG161 at doses of 2.97×10^7, 2.97×10^8 and 2.97×10^9 PFU/animal, respectively, showed no significant toxicity in cynomolgus monkeys, and the maximum tolerated dose (MTD) was greater than or equal to 2.97×10^9 PFU/animal.

Repeated dose toxicity study

VG161 was repeatedly administered to S.D. rats via intramuscular injection at doses of 1.54×10^7 and 1.54×10^8 PFU/mouse (daily, 5 days per week, for 4 weeks and a recovery period of 4 weeks) for 4 weeks. The dose was 3.3 and 32.9 times the lowest effective dose in rats for the low and high dose groups. The low and/or high dose groups showed no significant systemic toxicity during the treatment and recovery period, except for the acute phase reaction on the day after the first round of treatment and/or the day after the last dose. The no-observed-adverse-effect level (NOAEL) was considered 1.54×10^8 PFU/animal.

VG161 was administered to cynomolgus monkeys via intramuscular injection at a dose of 8.72×10^6, 2.57×10^7, and 2.57×10^8 PFU/mouse (daily, 5 days per week, for 4 weeks, and a recovery period of 4 weeks) for 4 weeks. The dose was 1, 2.9 and 29.4 times the lowest effective dose in monkeys for the low, medium and high dose groups. 14 animals for one group, half male and half female, were treated with single or multiple injections into the quadriceps femoris muscle of the hind limbs with the injection volume of no more than 0.5 ml per site. Under the experimental conditions, test article-related irritation was observed at the injection site and at the end of the 4-week recovery period, the irritation at the injection site did not fully resolve but showed a clear recovery trend. After the completion of the first round of administration (Day 6), a decrease in RBC (red blood cell count), HGB (hemoglobin concentration) and HCT (hematocrit) and Alb (serum albumin), as well as prolonged APTT (activated partial thromboplastin time), were observed compared with the same gender in vehicle control group during the same period. The above laboratory abnormalities did not cause any clinical toxicity and might be transient secondary changes. Specific IgG antibodies were detected in animals, but no immunotoxicity was observed. In the 2.57×10^8 PFU/animal dose group, VG161 DNA was detected in the whole blood before and after 5 doses, while at other time points and in each dose group, VG161 DNA was detected in the whole blood of only a few animals. On the day after the first round of administration (Day 6), the investigational drug in each dose group was mainly distributed in the muscle at the injection site and a small amount of the investigational drug was detected in the heart, spleen, spinal cord, left inguinal lymph node and sciatic nerve of individual animals in the 2.57×10^8 PFU/animal dose group. On the day after the last dose, a small amount of VG161 DNA was detected in the left inguinal lymph node, muscle at the injection site and/or spleen and small intestine of individual animals in the 2.57×10^7 and 2.57×10^8 PFU/animal dose groups. At the end of the 4-week recovery

period, only a small amount of viral DNA residue was detected in the muscle at the injection site of one animal in the 2.57×10^8 PFU/animal dose group, the viral level in the remaining tissues fell below the limit of quantification of the method. At all detection time points, the viral level in the reproductive system and other organs and tissues was below the limit of detection and no virus shedding occurred. During the experiment, the viral DNA was detected in the nasal secretions of one animal in the high-dose group on the day after one round of drugs (D6). The content was 2.10×10^2 copies/μg DNA. At other time points, the content of viral DNA in urine, feces and nasal secretion specimens was lower than the limit of detection, and no virus shedding occurred. No significant expression of the product was observed in the positive tissues and serum at each detection time point. Under the conditions of this experiment, VG161 was administered to cynomolgus monkeys by repeated intramuscular injection at the doses of 8.72×10^6, 2.57×10^7 and 2.57×10^8 PFU/animal for 4 weeks, respectively. No significant systemic toxicity was observed during the treatment period and recovery period in the low, medium and/or high dose groups, except for the acute phase reaction on the day after the first dose and/or the day after the last dose. The no-observed-adverse-effect level (NOAEL) was considered to be 2.57×10^8 PFU/animal.

Besides, a single injection of VG161 did not affect the central nervous system function of S.D. rats and a 4-week repeated injection did not affect the central nervous system, cardiovascular system and respiratory system functions of cynomolgus monkeys. The systemic active allergy test in guinea pigs and in vitro red blood cell hemolysis test in rabbits showed that allergic reaction and hemolytic reaction results were negative.

1.4 Pharmacokinetic and Biodistribution

Pharmacokinetic studies (see the Investigator's Brochure for details)

In male and female tumor-bearing mice (LS174T), a total of three absorption experiments of single intratumoral injection of VG161 were conducted to investigate the absorption kinetics of VG161 and the pharmacokinetic properties of the expressed product in serum. The experimental results showed that after a single administration of VG161 at a dose of 5.0×10^7 PFU/animal, the peak time T_{max} in the whole blood was 1-96 h and the peak concentration C_{max} was 51-1400 copies/μg DNA and the systemic exposure $AUC_{(0-t)}$ was 1616-211782 h*copies/μg DNA. The expression level of hIL-12 in the serum of animals increased after dosing, with the peak time T_{max} of 24 h, the peak concentration C_{max} of 0.83-4.20 ng/ml and afterwards gradually decreased and basically dropped near the limit of detection by D10. The serum level of hIL-15/IL-15Rα was above the limit of detection (0.79 to 4.27 ng/ml) at 24 h in males and between 24 and 96 h in females. The level of hIgG4 in the serum of female animals was above the limit of detection (81-88 ng/ml) between 1 and 24 h.

In male and female tumor-bearing mice (LS174T), a total of two VG161 multiple intratumoral injections (daily for 3 consecutive days, at an interval of 24 hours) were conducted to investigate the absorption kinetics of VG161 and the pharmacokinetic properties of the expressed product in serum. The experimental results showed that after multiple administrations of VG161 at a dose of 5.0×10^7 PFU/animal, with the peak time T_{max} in whole blood of 24-192 h after the last dose, the peak concentration C_{max} of 59 to 70 copies/μg DNA and the systemic exposure $AUC_{(0-t)}$ of 6392-15813 h*copies/μg DNA. The expression level of hIL-12 in the serum of animals increased after administration, with the peak time T_{max} of 1 h after the last dose, and the peak concentration C_{max}

of 1.11 ng/ml, then gradually decreased, and basically dropped near the limit of detection by D9. The level of hIL-15/IL-15Rα in serum was above the limit of detection (0.02 to 0.86 ng/ml) from 1 to 24 h after the last dose. The serum level of hIgG4 was above the limit of detection (2.1 to 35.28 ng/ml) from 1 to 48 h after the last dose.

The toxicokinetic study that was conducted in parallel with the "Toxicity study on a 4-week repeated intramuscular injection of VG161with a 4-week recovery period in Sprague-Dawley rats", the results showed that VG161 was administered by repeated intramuscular injection to S.D. rats at doses of 1.54×10^7 and 1.54×10^8 PFU/animal, respectively, once a day for 5 days a week (a total of 20 doses). After the first dose (D1), the concentration of VG161 DNA in the whole blood of animals in each dose group at each time point was lower than the limit of detection. After the last dose (D26), except that a lower level at 2 h post-dose was detected in only two male animals in the high-dose group, the level of VG161 DNA was below the limit of detection in all dose groups at each time point, indicating that the amount of viral DNA into the blood was relatively lower at the dose level used in this experiment. Serum expression product detection: hIL-12, hIL-15/IL-15Rα and hIgG4 of animals in the low and high dose groups were all below the limit of detection, and no significant expression product in the serum was observed at each detection time point. This may be explained by the fact that oncolytic virus was not replicated in normal cells or the amount of expression product was relatively low.

The toxicokinetic study that was conducted in parallel with the "Toxicity study on single intramuscular injection of VG161 in cynomolgus monkeys", the test results showed that a certain positive correlation was observed between the plasma concentration and the dose when VG161 was administered at a single dose of 2.97×10^7, 2.97×10^8 and 2.97×10^9 PFU/animal, respectively. After dosing, VG161 DNA was not detected in whole blood in the low-dose group. VG161 DNA was detected in whole blood of one female animal in the medium-dose group at 0.5 h post-dose, with the level of 54.04 copies/μg DNA. In the high dose group, the mean time to maximum concentration T_{max} in whole blood was 24 h, the maximum concentration C_{max} was 143.37-380.65 copies/μg DNA, and the systemic exposure $AUC_{(0\text{-}336h)}$ was 4427.43-47920.89 h*copies/μg DNA. The level of hIL-12 expression in the serum of animals in the high-dose group increased on D4 after dosing, then gradually decreased, and basically dropped near the limit of detection (0.156 pg/ml) by D15. The serum level of hIgG4 was slightly higher than the limit of detection in one female animal in the high-dose group on D8 and D15. The expression levels of hIL-15/IL-15Rα and hIgG4 in the other animal groups were below the limit of detection at each time point.

The toxicokinetic study that was conducted in parallel with the "Toxicity study on a 4-week repeated intramuscular injection of VG161 with a 4-week recovery period in Sprague-Dawley rats in cynomolgus monkeys", the results showed that VG161 was administered by repeated intramuscular injection to cynomolgus monkeys at doses of 8.72×10^6, 2.57×10^7 and 2.57×10^8PFU/animal, respectively, once a day for 5 days a week (a total of 20 doses). After the first dose (D1), VG161 DNA was detected in the whole blood of some animals in the low and high dose groups. After 5 doses (D5), a lower level of VG161 DNA in the whole blood was detected in individual animals in the low and medium dose groups, VG161 DNA before and after the dosing was detected in animals in the high-dose group. The level of VG161 DNA in whole blood increased at 0.5 h after dosing, reached a peak at 6 h after dosing, and then gradually decreased. The average level of VG161 DNA dropped to a low level at 24 hours after dosing, and the level of VG161 DNA

in whole blood at other time points in all dose groups was lower than the lower limit of quantification. At the last dose (D26), except that a lower level of VG161 DNA at individual time points was detected in only individual animals in the low and high dose groups after treatment, the content of VG161 DNA in whole blood at each time points was below the lower limit of quantification. A positive correlation was observed between the blood drug concentration and the dose. The serum level of hIL-15/IL-15Rα level was below the limit of detection in each group of animals at each time point. The 629.91 and 761.33 ng/ml levels in serum were detected in one animal in the high-dose group on D6 and D55. The hIgG4 level in serum of all animals in each group was below the limit of detection at each time point. The level of hIL-12 in serum was below the limit of detection in most animals during the study, which was not considered toxicologically significant for individual measurement, combined with the detection value obtained from vehicle controls and the animals themselves before treatment. Therefore, no significant expression of the product was observed in the serum at each detection time point.

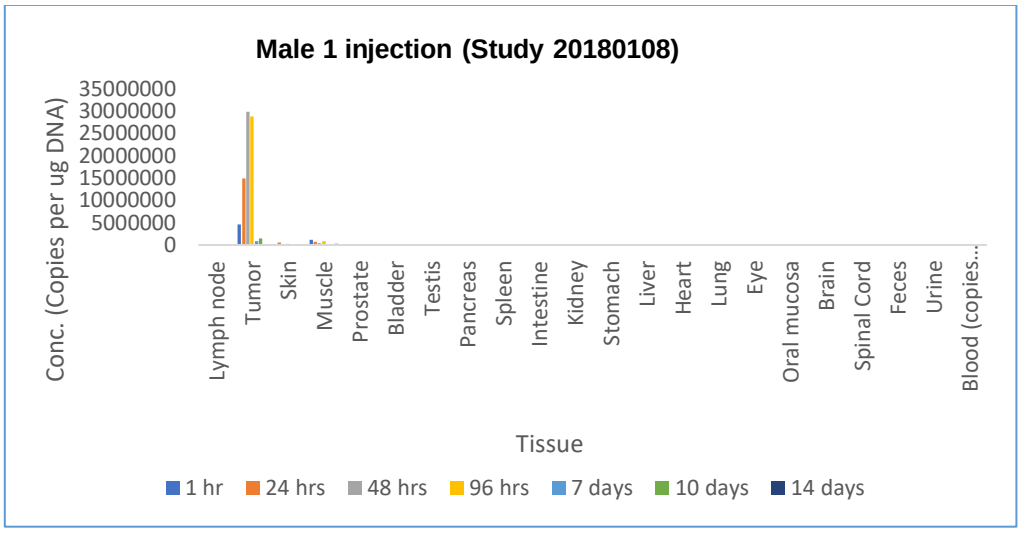
Biodistribution studies (see the Investigator's Brochure for details)

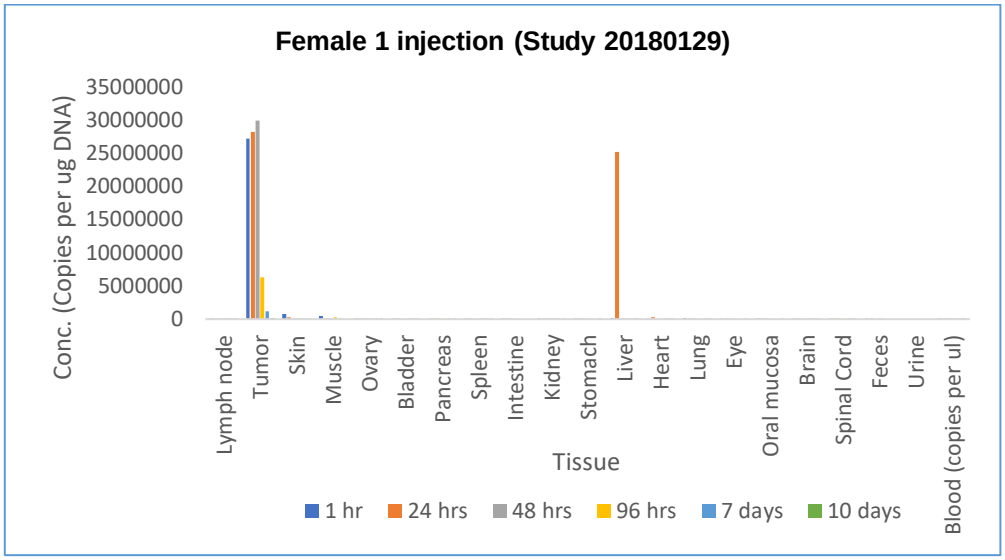
Single-dose biodistribution study in tumor-bearing mice:

In male and female tumor-bearing mice (LS174T), a total of three tissue distribution experiments with a single intratumoral injection of VG161 were conducted to investigate the tissue distribution and expression products of VG161.

The results of tissue distribution experiments showed that after a single administration of VG161 at a dose of 5.0×10^7 PFU/animal, the number of viral DNA copies in the tumor reached to the peak 48 h after injection, which was about 4000 to 5000 times of that in normal tissues (Figure 11). The different levels of VG161 DNA were also detected in peripheral tissues (skin, muscle and lymph nodes), liver, spleen and lung, but they were very significantly lower than those in tumor tissues. Within 10-14 days after dosing, VG161 DNA was detected in most tissues, and the level in tumor tissues was also significantly reduced, indicating that VG161 did not accumulate significantly. In the first distribution experiment in female tumor-bearing mice, the number of viral DNA copies in liver tissue was significantly increased in two animals at the 24 h time point, possibly due to the accidental injection of virus into the blood vessels at the injection site (Figure 11). For this reason, the distribution experiment was repeated and very little viral DNA was detected in the liver(Figure 11).

The results of the expression product experiment showed that the expression level of hIL-12 in the tumor increased significantly after intratumoral injection, with the peak time T_{max} of 24-48 h, and the peak concentration C_{max} of 146.03-830.07 ng/g. The expression level of hIL-15/IL-15Rα in tumors increased significantly after dosing, with the peak time T_{max} of 24-48 h, and the peak concentration C_{max} of 32.26-218.07 ng/g. The expression level of hIgG4 in the tumor increased significantly after administration, with the peak time T_{max} of 24 h, and the peak concentration C_{max} of 3318.21-4344.80 ng/g.





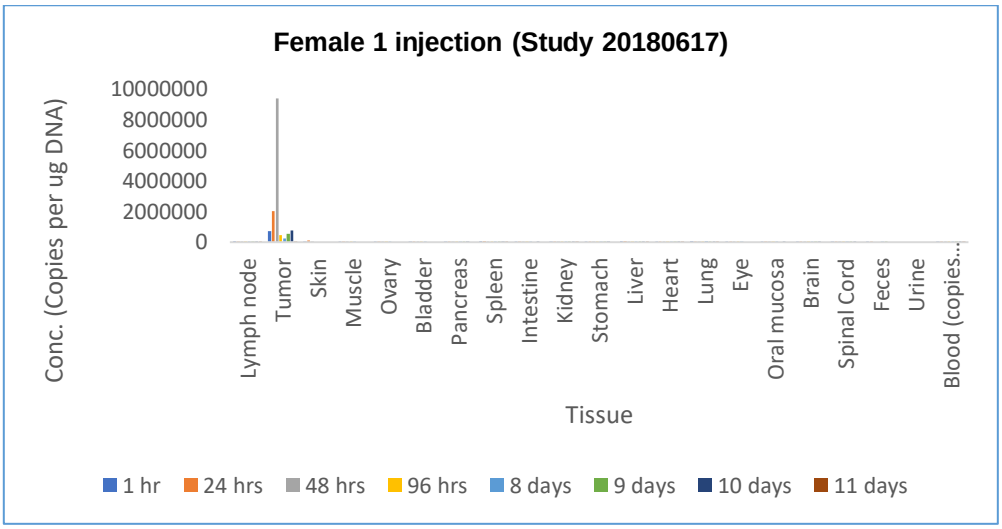


Figure 11: Tissue distribution of VG161 DNA in tumor-bearing mice (LS174T) after a single intratumoral injection of VG161 5.0×10^7 PFU/animal

Multiple Dose Biodistribution Study in Tumor-bearing Mice:

In male and female tumor-bearing mice (LS174T), two tissue distribution experiments with multiple intratumoral injections of VG161 were conducted to investigate the tissue distribution and expression products of VG161.

The results of tissue distribution experiments showed that after a single administration of VG161 at a dose of 5.0×10^7 PFU/animal, the number of viral DNA copies in the tumor reached to the peak from 1 to 24 h after injection, which was about 4,000 to 5,000 times of that in normal tissues (Figure 12). The different levels of VG161 DNA were also detected in peripheral tissues (skin, muscle and lymph nodes), liver, spleen and lung, but they were very significantly lower than those in tumor tissues. Within 10-14 days after dosing, VG161 DNA was not detected in most tissues, and the level in tumor tissues was also significantly reduced, indicating that VG161 did not accumulate significantly.

The results of the expression product experiment showed that the expression level of hIL-12 in the tumor increased significantly after intratumoral injection, with the peak time T_{max} of 1h post-dose, and the peak concentration C_{max} of 185.26 ng/g. The expression level of hIL-15/IL-15Rα in tumors increased significantly after dosing, with the peak time T_{max} of 1h post-dose, and the peak concentration C_{max} of 80.99 ng/g. The expression level of hIgG4 in the tumor increased significantly after dosing, with the peak time T_{max} of 1h post-dose, and the peak concentration C_{max} of 12797.68 ng/g.

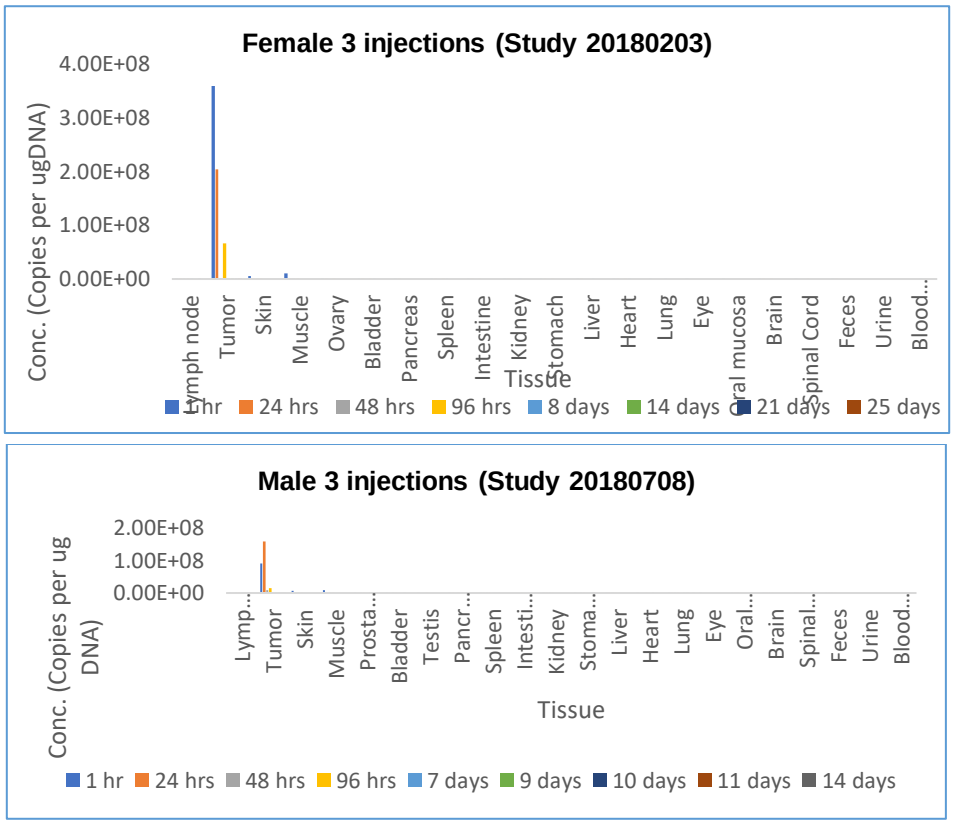


Figure 12: Tissue distribution of VG161 DNA in tumor-bearing mice (LS174T) after multiple intratumoral injection of VG161 5.0×10^7 PFU/animal

The toxicokinetic study that was conducted in parallel with the single and multiple intramuscular toxicology studies in rats and cynomolgus monkeys showed that: A small amount of VG161 DNA

was detected in spinal cord, sciatic nerve, inguinal lymph node, spleen and other tissues, but no relevant abnormal reactions were found combined with clinical observations of animals, and no treatment-emergent abnormal changes were found in histopathological examination. Therefore the distribution of VG161 in these tissues was not considered toxicologically significant.

1.5 Overview of clinical studies

The IP is classified into category 1 of therapeutic biologics.

As of 31 July 2022, four open-label, single-arm Phase I clinical studies were conducted for advanced malignant solid tumours and advanced primary liver cancer, an open-label, single-arm Phase IIa clinical study for advanced intrahepatic cholangiocarcinoma, and an open-label, single-arm, non-registered Phase II clinical study of VG161 in combination with nabulizumab for advanced pancreatic cancer. A total of 51 subjects were treated with VG161 injections, and no DLT was observed in any of the subjects.The increase in peripheral blood immunocytokines and tumour shrinkage in non-injected lesions after VG161 administration suggests that VG161 induces anti-tumour immune activation.

In the VG161-C102 trial, a total of 21 subjects were enrolled in the VG161-C102 trial as of 31 July 2022, and results of the effectiveness analysis are still being statistical. Of the 11 subjects in the dose-escalation period, a best overall response (BOR) of PR/iPR was observed in 1 case, a BOR of SD/iSD in 3 cases, a BOR of PD/iUPD in 6 cases, and a BOR of NE in 1 case; and in the 9 subjects in the treatment-expansion phase who had completed at least 1 post-baseline tumour assessment, a BOR of PR/iPR in 1 case, a BOR of PD/iUPD in 4 cases, and a BOR of PD/iUPD in 1 case, were observed. BOR was SD/iSD, and 4 cases had a BOR of PD/iUPD.

Of note, as of 31 July 2022, the best efficacy of SD/iSD was observed in a subject with hepatocellular carcinoma who had primary hepatocellular carcinoma (T3N0M1) administered at a dose level of 2 x 10^8 PFU per cycle (1 x 10^8 PFU once daily for two consecutive days), with a PFS of 10.7 months and an OS of >14.7 months, and is still being followed up In. A patient with intrahepatic cholangiocarcinoma had a best outcome of PR/iPR (C2D28-C5D28 tumour assessment resulted in PR/iPR), this subject had intrahepatic cholangiocarcinoma (T0N1M1) and was administered at a dose level of 3 x 10^8 PFU (1.0 x 10^8 PFU once a day for three consecutive days), with a PFS of 5.4 months and an OS of 7.7 months. A patient with hepatocellular carcinoma (T0N1M1) had a best outcome of PR (PR/iPR on C4D28 tumour assessment) and was administered at a dose level of 3 x 10^8 PFU (1.0 x 10^8 PFU once a day for three consecutive days), and is currently being followed up.

In the VG161-A101 trial, as of 31 July 2022, the best efficacy of SD/iSD was observed in a chondrosarcoma patient with chondrosarcoma (T2N0M1) administered at a dose level of 2.5 x 10^8 PFU per cycle (5 x 10^7 PFU/day, administered for 5 days per cycle), with a PFS of >9 months. This subject is still in Follow-up.

In the VG161-IIS-201 trial, as of 31 July 2022, the best efficacy of PR/iPR was observed in a patient with pancreatic cancer in a subject with pancreatic cancer (T2N0M1) who was administered dose levels of VG161 at 2.0 x 10^8 PFU per cycle (1 x 10^8 PFU/day, administered on

Days 1 and 2 of each cycle) and nabulizumab administered on Days 1 and 2 of each cycle. 3 mg/kg per dose administered on days 8 and 22 of the cycle.

Similar products at home and abroad

Oncolytic viruses have officially entered clinical trials since the 1990s, in which the number of participants has exceeded 4,000. Up to now, the clinicaltrials.gov database shows that nearly 30 oncolytic virus products have entered various phases of clinical trials worldwide and used for more than 70 treatment regimens. Many products have entered phase III clinical trials in addition to the listed products.

Based on the available clinical data and clinical trial data, oncolytic virus therapy exhibits good safety and tolerability. The main adverse reactions are: transient fever, chills, fatigue, tachycardia and change in blood pressure (such as hypotension or hypertension) (rare) after injection, etc. The most common adverse reactions are mainly rapid and transient flu-like symptoms. The FDA has issued guidance documents on virus treatment (FDA-2014-D-0852, Design and Analysis of Shedding Studies for Virus or Bacteria-Based Gene Therapy and Oncolytic Products)。

Herpes simplex virus is currently the most studied, and is also one of the oncolytic viruses with the best anti-tumor effect. The oncolytic virus T-Vec from Amgen (US) has shown good results in Phase III clinical trials. In 2014, Amgen submitted the marketing application of the first oncolytic herpes virus treatment of malignant melanoma to the European Medicines Agency and the US FDA. The US FDA expert committee voted to support the marketing application in May 2015. Since then, T-Vec has been approved by the FDA and EMA.

In addition to T-Vec, which has been approved for marketing, nearly ten oncolytic HSVs have entered the clinical study Period, such as: HSV1716, G207, HF10, NV1020 (R7020), etc. (see Table 1). At present, 4 kinds of oncolytic herpes simplex virus with anti-tumor effect have obtained IND registration approval from CFDA/NMPA, including 1) the recombinant human GM-CSF Herpes Simplex Virus Injection (OrienX010) (CTR20171275/20180505) produced by Beijing OrienGene Biotechnology Ltd., 2) recombinant type II herpes simplex virus injection preparation (OH2) (CTR20182222) produced by Wuhan Binhui Biotechnology Co., Ltd., 3) recombinant herpes oncolytic virus(T3011) (CXSL1900042) simultaneously carrying both PD-1 antibody and IL-12 produced by Immvira Pharma Co., Limited, and 4) recombinant herpes oncolytic virus (CXSL1900078) carrying with PD-1 antibody produced by Zhejiang Yangshengtang Biotechnology Co., Ltd. All the products mentioned above are undergoing phase I and II clinical trials. Existing clinical trial data have confirmed the safety of the oncolytic herpes simplex virus.

Table 1: Overview of foreign literature reports on clinical trials of herpes simplex virus-related products

Oncolytic virus	Indication	Trial phase	Combination therapy or not	Main results of the trial
Talimogene Laherparepvec	Malignant melanoma	III	None	DRR: 16.3%; ORR: 26.4%; OS: 23.3 G3 or 4 AE: cellulitis (2.1%) (Andtbacka RH, et al. J Clin Oncol. 2015)
	Malignant	II	Ipilimum	ORR: 39% (vs. 18% in ipilimumab arm); AE: fatigue

	melanoma		ab	(59%), chills (53%), diarrhea (3%) (Chesney J, et al. J Clin Oncol. 2018)
	Malignant melanoma	Ib	Ipilimumab	No DLT; G3/4 AE:26.3% ORR: 50%; DRR (≥ 6 mon): 44%; PFS (18 mon): 50%; OS (18 mon): 67% (Puzanov I, et al. J Clin Oncol. 2016)
	Malignant melanoma	IIIb	None	G3/4 AE: 7.3%; 1 of 41 (2%) patients tested positive for T-vec DNA by qPCR at injected lesion (Chesney J, et al. Melanoma Res. 2018)
	Malignant melanoma	Ib	Pembrolizumab	ORR: 62%, CRR: 33%; G3/4 AE: rush (10%), diarrhea (5%) (Ribas A, et al. Cell 2017)
HSV1716	Pediatric oncology	I	None	No DLT, G1/2 AE: fever, chills, cytopenia; No response (Streby KA, et al. Clin Cancer Res. 2017)
	Pediatric oncology	I	None	IV. No DLT; No response (Streby KA, et al. Mol Ther. 2019)
	Oral squamous cell carcinoma	NA	None	No AE; No response (Mace AT, et al. Head Neck. 2008)
G207	Malignant glioma	I	None	TTP: 3.5 mon; No toxicity or SAE related to IP (Markert JM, et al. Gene Ther. 2000)
	Malignant glioma	I	Radiotherapy	SAE related to IP: seizures (33.3%); DCR: 66.7%; OS: 7.5 mon (Markert JM, et al. Mol Ther. 2014)
	Glioblastoma multiforme	Ib	Surgery	G3/4 AE related to IP: 10 in 5 patients; TTP: 3 mon, No response (Markert JM, et al. Mol Ther. 2009)

HF10	Pancreatic cancer	I	None	No IP related SAE; ORR: 33%, PFS: 6.3 mon, OS: 15.5 mon (Hirooka Y, et al. BMC Cancer 2018)
	Advanced tumors	I	None	Japanese trial; No SAE (Kasuya H, et al. Hepatogastroenterology. 2014)
	Pancreatic cancer	Pilot	None	Intraoperative injection; No AE; PR: 1 of 6 (Nakao A, et al. Cancer Gene Ther. 2011)
NV1020	Colorectal cancer metastases to liver	I/II	None	G3/4 AE: lymphopenia (2/31, 6.3%) ORR: 1/32 (3.1%), TTP: 6.3 mon, OS: 11.8 mon (Geevarghese SK, et al, Hum Gene Ther. 2010)
	Colorectal cancer metastases to liver	I	None	SEA in 3 patients (an increase of GGT, diarrhea, and leukocytosis) (Kemeny N, et al. Hum Gene Ther. 2010)
	Colorectal cancer metastases to liver	I	Local Chemotherapy	Hepatic arterial administration; 39% and 20% reduction of tumor size in 2 patients (Fong Y, et al. Mol Ther. 2009)
	Colorectal cancer metastases to liver	I/II	None	SAE: 5/32 (15.63%) (Sze DY, et al. Hum Gene Ther. 2012)

1.6 Benefit and risk assessment

Clinical studies have demonstrated the anti-tumor effect of HSV-1 oncolytic virus over the years. The IP VG161 strengthens the activation of local T cells and NK cells by carrying IL12/IL15/IL15RA gene and PD-L1 blocking peptide on the original oncolytic virus vector. It is expected to exert more potent anti-tumor effect through synergistic effect of immune stimulation. Preclinical in vitro and in vivo pharmacodynamic tests also suggest that VG161 has a significant inhibitory effect on multiple tumor cell lines and their transplanted tumor models, especially liver cancer mouse transplanted tumors. Therefore, it may bring potential therapeutic benefits to patients with advanced liver cancer.

There is currently no report of a large amount of human research data on VG161. In the ongoing phase I dose-escalation clinical study conducted in Australia, the single dose has reached 2.0 x 10^8 PFU. No DLT and related SAEs(except fever) have been found so far, The clinically known suspected serious adverse reaction was fever that could be completely recovered. . In pre-clinical acute and chronic toxicology tests conducted in rats and cynomolgus monkeys, no significant toxicity was observed except for local injection reactions. In clinical studies on similar drugs, common toxic reactions of HSV-1 oncolytic virus included flu-like symptoms, reactions at injection site and HSV-1 infection; common toxic reactions associated with immunotherapy were immune-related adverse events (irAEs) occurring in multiple systems such as skin, gastrointestinal tract, liver, endocrine, lung and musculoskeletal system. Most irAEs were mild to moderate in severity, and the incidence of severe irAEs was low.

In terms of risk control, subjects will be excluded if they have a high anticipated safety risk at screening, including but not limited to: subjects who experienced ≥ Grade 3 irAEs from prior immunotherapy, had active or potentially recurrent autoimmune disease, had a negative herpes

simplex virus type Iantibody test result, were in the stage of recurrent herpes simplex virus infection, or had other serious medical conditions. The criteria for selection of subjects are detailed in Section 3 (3 Subject selection).

Subjects were closely monitored for safety parameters, including vital signs, physical examination, laboratory tests such as hematology, urinalysis, blood biochemistry, coagulation function, thyroid function, and auxiliary tests such as 12-lead ECG, during the treatment period and 28 days after the last dose of IP. Meanwhile, the occurrence of adverse events from the time of signing the informed consent form to 28 days after the last dose, and adverse events whose causality to the IP could not be ruled out 28 to 90 days after the last dose were closely monitored and recorded. Serious Adverse Events (SAE) and Suspected Unexpected Serious Adverse Reactions (SUSAR) will be reported in an expedited fashion following relevant laws and regulations.

In terms of toxicity management, the main expected toxicities include flu-like symptoms, injection-site reactions, HSV-1 infection and irAE. Moreover, because VG161 carries IL12/IL15/IL15RA genes and exerts synergistic immunostimulatory effects by expressing cytokines, the occurrence of Cytokine Release Syndrome (CRS) after dosing should also arouse vigilance. A detailed toxicity management plan has been established in this protocol and described in Section 6.3 (6.3 Handling of expected toxicity and dose modification rules).

In terms of biosafety, a detailed biosafety protection plan (6.4 Biosafety protection) is formulated in this study protocol.

For safety and pharmacovigilance, the sponsor has established a professional team to assort, evaluate and analyze adverse events, and promptly notify the investigator when significant or unexpected safety risks occur, and discuss with the investigator at the meeting if necessary. The sponsor will promptly notify the investigator of any update on other safety or toxicology data during the study.

2 Main Objectives

2.1 Single-agent dose-escalation period (period 1)

2.1.1 Primary objective(s)

The primary objective of the study is to evaluate the safety and tolerability of single and multiple intratumoral injections of VG161 under ultrasound guidance to treat patients with primary liver cancer who have failed standard treatments and to explore the maximum tolerated dose (MTD) of VG161, and determine the recommended clinical administration dose.

2.1.2 Secondary objective(s)

1) To evaluate the pharmacokinetic (PK) profile and virus excretion of single and multiple intratumoral injections of VG161;
2) To evaluate the preliminary efficacy of VG161 monotherapy;
3) To monitor changes in immune measures related to the pharmacodynamics of VG161.

2.2 Monotherapy extension period (period 2)

2.2.1 Primary objective(s)

To evaluate the efficacy of multiple intratumoral injections of VG161 under ultrasound or CT guidance in treating patients with advanced primary liver cancer. The main observation index is the objective response rate (ORR).

2.2.2 Secondary objective(s)

1) To evaluate the secondary observation indicators of the effectiveness of VG161;
2) To further evaluate the safety of VG161;
3) To monitor changes in immune measures related to the pharmacodynamics of VG161.

3 Subject selection

3.1 Inclusion criteria

1. Males or females aged, 18 to 75 years, inclusive.
2. According to 'The Diagnostic and Therapeutic Criteria for Primary Liver Cancer' (2019 Edition), subject with advanced primary hepatocellular carcinoma, intrahepatic cholangiocarcinoma, combined hepatocellular which is refractory/relapsed after and/or intolerant of standard therapies or for which no standard therapy exists. For the second period of Simon's two-period approach in the Monotherapy extension period, only patients with advanced primary hepatocellular carcinoma were enrolled.
3. There are tumor lesions intrahepatic and / or extrahepatic metastases that can be injected under B ultrasound or CT guidance and meet the volume requirements of the current dose group (the injected lesions are preferably the main tumor burden lesions, see 6.2 Route of administration and method of use for the selection of injectable lesions), and the longest diameter of injectable tumor lesion >1.5cm (or the shortest diameter of lymph node lesions)
4. Eastern Cooperative Oncology Group (ECOG) scores 0 or 1.
5. Life expectancy is at least 3 months.
6. Required organ function:
   1) Hematology (no blood transfusion or colony-stimulating factor treatment within 14 days): ANC≥1.5×10^9/L, PLT≥75×10^9/L, Hb≥85g/L;
   2) Liver function: TBIL ≤ 1.5 × ULN, ALT ≤ 5 × ULN, AST ≤ 5 × ULN;
   3) Child-Pugh A-B level;
   4) Renal function: Cr ≤ 1.5 × ULN and creatinine clearance ≥ 45 ml/min (calculated according to Cockcroft-Gault formula);
   5) Coagulation function: activated partial thromboplastin time (APTT) ≤1.5 × ULN, prothrombin time (PT) ≤1.5 × ULN, international normalized ratio (INR) ≤1.5 × ULN.
7. HBV-DNA is negative . Subject with positive HBV-DNA must follow 'Guidelines for the prevention and treatment of chronic hepatitis B' (2019 Edition) or clinical routine for treatment.
8. Subjects of childbearing potential (male and female) must agree to use a reliable contraceptive method (hormone or barrier method or abstinence) during the study and for at least 90 days following the last dose; females of childbearing potential must have a negative blood pregnancy test within 7 days of study enrollment.

9. Subjects are required to give informed consent to the study prior to the trial and voluntarily sign a written informed consent form.

3.2 Exclusion criteria

Subjects are to be excluded from the study if they meet any of the following criteria:

1. Subject in prior anti-tumor therapies such as chemotherapy, radiotherapy, biotherapy, endocrinotherapy, targeted therapy, immunotherapy within 4 weeks of study treatment initiation, including oral fluorouracil and small molecule targeted drugs within 2 weeks before the first dose of IP (Investigational product) or within 5 half-lives of the drug (whichever is longer).
2. Transcatheter arterial chemoembolization(TACE) within 4 weeks of study treatment initiation
3. Participation in clinical trials of any other investigational agents within 4 weeks of study treatment initiation.
4. Major organ surgery (excluding puncture biopsy) or significant trauma within 4 weeks of study treatment initiation.
5. Patients who received systemic treatment with either corticosteroids (>10 mg/ daily prednisone or equivalent) or other immunosuppressive medications within 14 days of study treatment initiation. Patients who have received systemic use of corticosteroids (prednisone > 10 mg/day or equivalent dose of products in the same therapeutic class) or other immunosuppressive agents within 14 days before the first dose of IP, with the exception of the following: use of topical, ocular, intra-articular, intranasal and inhaled corticosteroids; short-term use of corticosteroids for prophylaxis (e.g., prevention of contrast media allergy).
6. Adverse reactions of previous anti-tumor therapy have not recovered to CTCAE 5.0 grading ≤ 1 (except for alopecia and other toxicities that are not considered a safety risk by the investigator).
7. Subjects with central nervous system metastases, or spinal cord metastases..
8. Negative results of herpes simplex virus type I antibody test (both HSV-1 IgG and HSV-1 IgM).
9. Being in a period of recurrent herpes simplex virus infection with corresponding clinical manifestations such as herpes orofacialis, herpetic keratitis, herpetic dermatitis and genital herpes.
10. Other uncontrolled active infections.
11. History of immunodeficiency, including positive HIV antibody testing.
12. History of severe cardiovascular disease:
    1) Ventricular arrhythmia requiring clinical intervention;
    2) QTc interval > 480 ms;
    3) Acute coronary syndrome, congestive heart failure, stroke or other cardiovascular events of grade III and above within 6 months prior to the first dose of study drug;
    4) New York Heart Association (NYHA) classification ≥ Class II or left ventricular ejection fraction (LVEF) < 50%;
    5) Uncontrolled hypertension. (systolic blood pressure≥150 mmHg, or diastolic blood pressure≥100 mmHg after treatment).
13. Active or previous autoimmune diseases that may relapse (such as systemic lupus erythematosus, rheumatoid arthritis, vasculitis, etc.), except clinically stable autoimmune thyroiditis.
14. Previous immunotherapy with an immune-related adverse event (irAE) such as immune-related pneumonia,

myocarditis, etc., which, in the judgement of the investigator, may affect the safety of the trial medication.

15. Known alcohol or drug dependence.
16. Subjects with mental disorders or poor compliance.
17. Females during pregnancy or breastfeeding.
18. Subjects who, in the opinion of the Investigator, have other serious systemic diseases or are otherwise unsuitable for participation in this clinical study.

3.3 Criteria for Early Termination

1. Evidence of disease progression and, in the opinion of the investigator, the subject is not deriving clinical benefit from continuing study treatment.
2. Any of the following drug-related adverse events, assessed by the investigator to be possibly, probably, or definitely related to the drug:

   (1) Haematological toxicity

   -Grade 4 neutropenia lasting > 7 days.

   -≥ Grade 3 febrile neutropenia (single measurement of temperature ≥ 38.3 ° C or ≥ 38.0 ° C for 1 hour with concomitant absolute neutrophil count [ANC] < 1000/mm3)

   -Grade 4 Thrombocytopenia

   -Grade 3 thrombocytopenia with haemorrhage

   -Grade 4 anaemia

   (2) Non-haematological toxicity

   -Grade 4 non-haematological toxicity

   -Failure of the following symptoms to resolve to ≤ Grade 2 during treatment 7 days after administration: Grade 3 influenza-like symptoms (e.g., fever, chills, fatigue, body aches or pains, anorexia, dry cough), Grade 3 injection site reactions

   -Grade 3 CRS of > 72 hours duration or Grade 4 CRS of any duration

   -Any Grade 3 or higher non-haematological toxicity that meets the pre-specified criteria for being at least likely to be caused by the test drug (exclude Grade 3 fever, Grade 3 injection site pain, asymptomatic laboratory test abnormalities at the investigator's judgement).

   -Grade 4 immune-related adverse events

   - > Grade 3 colitis or non-infectious pneumonia or myocarditis of any grade

   (3) Other toxic reactions to the study drug that the investigator determines require permanent discontinuation of the drug.

   (4) Any Grade 5 AE.

   The above adverse events were graded according to the NCI CTCAE version 5.0.

3.4 Criteria for Subject Withdrawal

1) According to GCP and ICH guidelines, all subjects have the right to withdraw from the study at any time during the trial.
2) Major deviation or protocol violation with impact on tolerability, safety, or pharmacokinetic

evaluation of the drug.

3) Withdrawal as determined by the investigator.

3.5 Subject Replacement

Eligible subjects should be selected for the replacement to complete the trial in the following conditions:

During the dose-escalation study, the subjects are not considered for the overall DLT assessment in the dose level cohort and should be replaced if they fail to complete the observation in Cycle 1 due to reasons other than DLT, or received less than 80% of the scheduled dose in Cycle 1 for reasons other than DLT (e.g., those without DLT who have to discontinue the treatment due to injection site reaction). These replaced subjects are included in the Safety Set (SS) for statistics, but not included in the total number of subjects in the cohort when calculating the occurrence proportion of DLT events.

4 Study Design

This trial is an open-label, dose-escalation, preliminary efficacy phase I clinical study in patients with advanced liver cancer who have failed standard therapy.

4.1 Escalation Rules

4.1.1 Single-agent dose-escalation period (period 1)

4.1.1.1 Estimation of initial dose

VG161 is an oncolytic virus drug. The starting dose of its phase I clinical trial in humans follows the requirement of the Technical Guidelines for Clinical Trials of Anti-tumor Drugs (National Medical Products Administration, issued on May 15, 2012). The dose was determined based on "For non-cytotoxic drugs, the starting dose of phase I clinical trials can be calculated using 1/5 or higher of the NOAEL of non-rodents in non-clinical trials."

The pre-clinical acute toxicity test of VG161 showed that no significant toxicity was observed at a single dose of 4.5×10^8 PFU in rats and 3×10^9 PFU in monkeys.

In the pre-clinical 28-day repeated dose chronic toxicity test in rats (a cycle consisting of a 5-d successive treatment period and a 2-d washout period, a total of 4 cycles) (weight of rats: 200g), the NOAEL was 1.54×10^8 PFU/animal (male or female).

In the pre-clinical 28-day repeated dose chronic toxicity experiment in cynomolgus monkeys (a cycle consisting of a 5-d successive treatment period and a 2-d washout period, a total of 4 cycles) (weight of monkeys:3 kg), the NOAEL was 2.57×10^8 PFU/animal (male or female).

No significant toxic and side effects were observed in the preclinical chronic toxicity test, and some mild reactions were clinically controllable and transient, which quickly resolved. Cynomolgus monkeys have a more direct and significant response to VG161, so they are considered a more sensitive species. Therefore, based on the results of the NOAEL of the 28-day repeated dose chronic toxicity test in cynomolgus monkeys, considering a safety factor of 10, the

clinical initial dose (HSD) of VG161 was calculated to be 1.66×10^8 PFU/person by average weight of a patient of 60 kg (Table 2).

Table 2: NOAEL and calculation (HED) of chronic toxicity test in rats and monkeys

	Dose level (NOAEL)	HED (60 kg human)	HSD (1/10 HED)
Rat (200g)	1.54×10^8 PFU/animal	7.44×10^9 PFU/person	7.44×10^8 PFU/person
Monkey (3 kg)	2.57×10^8 PFU/animal	1.66×10^9 PFU/person	1.66×10^8 PFU/person

In the ongoing phase I dose-escalation clinical trial in Australia, a single dose 3-dose level escalation study has been carried out, including 5.0×10^7 PFU, 1.0×10^8 PFU and 2.0×10^8 PFU. No DLTs and related SAEs were observed. The trial is ongoing.

Combined with the above data, considering the safety of subjects and potential clinical benefits, the initial dose of this trial was set to 1.0×10^8 PFU/time.

4.1.1.2 Determination of maximum dose-escalation

In the preclinical 28-day repeated dose chronic toxicity test (a cycle consisting of a 5-d successive treatment period and a 2-d washout period, a total of 4 cycles) in cynomolgus monkeys, no significant toxicity was observed at the highest dose of 2.57×10^8 PFU/animal, which corresponded to a HED of 1.66×10^9 PFU/person. The highest dose to be explored in the tentative escalation period of this study is 5.0×10^8 PFU once daily for 3 days.

4.1.1.3 Method and frequency of administration

The development of oncolytic virus drugs is mainly based on its mechanism of specific replication in tumors leading to tumor cell lysis without harming normal cells. Therefore, intratumoral injection is a common method of administration of oncolytic virus drugs. At present, the United States has approved the oncolytic virus T-Vec for treatment of malignant melanoma via intratumoral injection, and other oncolytic viruses in clinical trials are also mainly administered via intratumoral injection. Therefore, VG161 in this trial was administered via intratumoral injection.

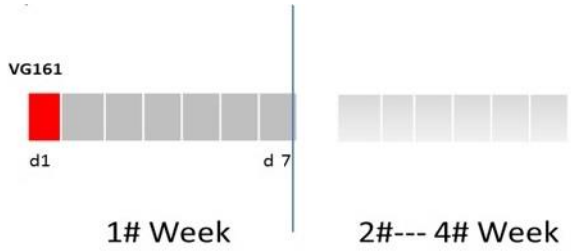
The regimen of VG161 injection is tentatively proposed as once daily in per cycle, for 1-3 days, for every 28 days as a dosing cycle, based on the following: 1) the administration method of intratumoral injection is limited by the final volume of injection; 2) the clinical trials and post-marketing clinical applications of the similar drug T-Vec; 3) the maximum volume that can be tolerated by local injection in the liver; 4) the preclinical pharmacodynamic experimental data of VG161; 5) the more significant efficacy with continuous multiple administrations at medium doses, and 6) the above-mentioned pharmacodynamic and clinical operability.

After subjects are enrolled in the dose-escalation period, they will enter different dose groups in sequence to receive VG161 administration. The investigational drug received by the first dose group is a single intratumoral injection. The total dose is given in days from the second dose group, and the maximum intratumoral injection dose per day is 1.7×10^8 PFU (the drug injection volume is about 3.3 ml), and the dose duration was increased from 2 consecutive days to 3 consecutive days. The DLT observation period was 28 days after the first dose. In the single intratumoral injection or consecutive treatment for 2 to 3 days, subject have completed the safety assessment

during the DLT observation period of the first cycle and the safety should be confirmed, according to the investigator's determination and the principles of ethical medication and upon discussion and decision by the SRC. Only then, the subject can continue taking the IP at the same dose as that in the first cycle for a cumulative period of no more than 4 cycles (each cycle consisting of 28 days) until disease progression (iRECIST criteria, iCPD), no benefit from continuing treatment with the IP any more in the opinion of the investigator, or intolerable toxicity, or termination of the study for other reasons. If a subject achieves stable disease or partial response after 4 cycles, the SRC may decide whether to continue the treatment after discussion.

During the study, the method or frequency of administration may be changed depending on the available safety and PK data.

A graphical representation of the single-day dose group (1.0×10^8 PFU) is provided below:



The 2 to 3 consecutive days dosing groups (2.0×10^8 PFU to 5.0×10^8 PFU) are shown in the figure below:

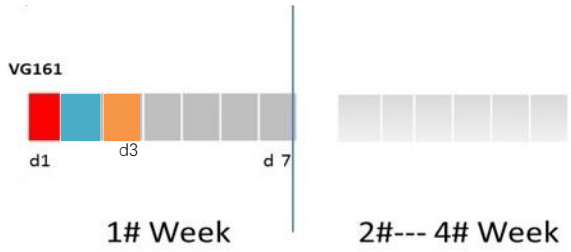


Figure 13: Dosing schedule

4.1.1.4 Dose-escalation group settings

The initial dose of VG161 is set at 1.0×10^8 PFU and the predetermined maximum dose is 5.0×10^8 PFU. If the dose is safe and well-tolerated, the investigator and sponsor will consider the feasibility of local injection, jointly discuss and decide whether to try an even higher dose. During the trial, exploratory intermediate-dose or frequency adjustments may be introduced based on the safety and PK data. The dose group settings for the specific dose-escalation study are shown in Table 3.

Table 3: Dose group settings for the dose-escalation study

Dose groups	Total dose of VG161 administered per cycle	Predetermined total drug volume [a] (predetermined at drug concentration 5×10^7 PFU/ml)	Mode of Injection[a, b] (predetermined at a drug concentration of 5×10^7 PFU/ml)	Number of subjects[c]
1	1.0×10^8PFU	2ml	Single-day	1
2	2.0×10^8PFU	2ml x 2 days	1.0×10^8 PFU daily for 2 days	1
3	3.0×10^8PFU	2ml x 3 days	1.0×10^8 PFU daily for 3 days	3
4	4.0×10^8PFU	2.7ml x 3 days	1.3×10^8 PFU daily for 3 days	3
5	5.0×10^8PFU	3.3ml x 3 days	1.7×10^8 PFU daily for 3 Days	3

a. The specification of the titer is $\geq 1 \times 10^7$ PFU/ml. The actual administration volume of the drug is calculated based on the labeled value indicated by the manufacturer. The actual volume may be higher or lower than the total volume of the drug predetermined at a drug concentration of 5×10^7 PFU/ml. It is recommended that the investigator determine the injection volume based on the size of the subject's lesions. Dilution with saline is allowed and injection of the total dose into multiple lesions is allowed.

b. In the continuous multiple injection dose group, if it is difficult to re-inject the injected lesions, if the subject has multiple injectable lesions according to the judgment of the investigator, other lesions can be injected for subsequent treatment, or it can be injected every other day. (See 6.2 Route of administration and method of use for details).

c. A principle of accelerated titration combined with a 3+3 dose escalation is used. For the specific dose-escalation principle and possible number of cases, refer to the dose-escalation principle.

4.1.1.5 Guidelines for dose escalation

The intergroup dose escalation will be based on the occurrence of DLTs in Cycle 1.

The dose-escalation period uses the principle of accelerated titration combined with 3+3 dose escalation. Dose Group 1 and Dose Group 2 use the accelerated titration design, and Dose Group 3 to Dose Group 5 use the 3+3 design. Whether it is accelerated titration or 3+3 method in the dose-escalation period, at the escalation decision point of each dose group, the safety review committee will discuss whether to escalate to the next dose based on the results of the previous group.

Safety Review Committee (SRC): During the study period, a Safety Review Committee (SRC) consisting of the Principal Investigator, Contract Research Organization (CRO) representatives (medical monitors and other relevant personnel), and sponsor representatives will be established to review safety, PK, efficacy data generated from the study, to determine the dose level and dosing regimen of each dose escalation in the dose-escalation study, to determine the MTD, and to determine the recommended dose. The SRC will also decide whether to add an unscheduled dose for testing.

Starting from the accelerated titration dose group, one subject will be enrolled first. If the subject does not develop Grade 2 or higher toxicity related to the IP (except fever, flu-like symptoms, injection site reaction, injection site pain), the safety of the dose will be discussed and evaluated by the Safety Review Committee before escalation to subsequent dose groups. If the subject experiences Grade 2 or higher toxicity that is definitely or probably related to the test sample (except pyrexia, flu-like symptoms, injection site reactions, injection site pain), the subject will switch to a 3+3 dose-escalation approach starting from that dose level. The dose ascending design should be consistently implemented or appropriately modified according to the recommendations as reviewed and evaluated by the Safety Review Committee. For example, the 3+3 dose-escalation method is adopted from Dose Group 2 ahead of schedule if necessary. If the first two dose groups

are not switched to the 3+3 dose-escalation method, dose group 3 cannot start the 3+3 design study until the safety data of the first two dose groups have been comprehensively discussed and analyzed by the Safety Review Committee after completion of DLT observation of Dose Group 2. The 3+3 dose-escalation approach will be used starting from Dose Group 3. Three patients will be enrolled first; if no DLT occurs, the next dose group will be tested. If 2 or more of the 3 patients experience a DLT, the dose escalation will be discontinued. If 1 out of 3 patients experiences a DLT, 3 additional patients will be enrolled in this group. If 1 or more of the additional 3 patients still experience a DLT, the dose escalation will be discontinued. If the additional 3 patients do not experience a DLT, the dose will be escalated to the next group. If Dose Group 3 needs to discontinue dose-escalation, termination of the study will be considered.

4.1.1.6 Dose-limiting toxicity (DLT)

A DLT is defined as any of the following toxicities that are related to the IP (including related, probably related, or possibly related) occurring within 28 days of the first cycle of treatment:

(1) Haematological toxicity:

- Grade 4 neutropenia lasting > 5 days;
- ≥ Grade 3 febrile neutropenia (ANC < 1000/mm^3 with a single temperature measurement > 38.3°C or sustained temperature ≥ 38.0°C for more than 1 hour);
- Grade 4 thrombocytopenia;
- Grade 3 thrombocytopenia with bleeding;
- Grade 4 anemia;

(2) Non-haematological toxicity:

- Grade 4 non-haematological toxicity;
- Grade 3 flu-like symptoms (e.g., fever, chills, fatigue, body aches or discomfort, anorexia, dry cough) that cannot be relieved to ≤ Grade 2 after 7 days of treatment, grade 3 injection site reactions and grade 3 cytokine release syndrome (CRS);
- Other Grade 3 non-hematological toxicities (except Grade 3 fever and Grade 3 injection site pain), which cannot be relieved to ≤ Grade 2 within 3 days after the best supportive care, except asymptomatic laboratory abnormalities as judged by the investigator;

(3) Other toxic reactions that require permanent discontinuation as determined by the investigator.

The above adverse events are graded according to the National Cancer Institute Common Terminology Criteria for Adverse Events (NCI CTCAE) version 5.0.

4.1.1.7 Maximum tolerated dose (MTD)

The MTD is defined as the dose at which ≤1/6 of subjects in the current dose group experience a DLT, and ≥33% of subjects in the next higher dose group experience a DLT (i.e., 2/3 or 2/6). For the MTD group, it is necessary to have at least 3 subjects with the completed safety assessments of the first cycle and obtain adequate PK data. If no DLT was observed among the 3 subjects enrolled in the highest dose group, the MTD and the recommended dose for the extension period were to be determined by the SRC.

4.1.2 Monotherapy extension period (period 2)

After determining the recommended dose in the single-agent dose-escalation period, subjects will enter the extension period. This part is a single-agent, single-dose and single-arm design, and the indication is advanced primary liver cancer. The dose is the recommended dose recommended in the first period (including the number of continuous administrations). Simon's minimal maximum two-period design (Simon 1989) is applied, and the type I error rate α=0.05 and statistical power 1-β=0.85 are pre-specified; Assuming a historical control ORR of 11% (referring to the 2019 version of the primary liver cancer diagnosis and treatment specifications, regorafenib is recommended as a second-line treatment, and its data comes from a RESORCE study, which included 573 Child-Pugh A advanced liver cancer patients with ineffective sorafenib treatment, based on mRECIST criteria, the ORR of the regorafenib treatment group was 11%, the DCR was 65%, the median OS was 10.6 months, and the PFS was 3.1 months). The ORR with VG161 was 20% higher than the historical control at 31%. The trial was designed according to Simon's two-period approach and was conducted in two periods; in the first period, tumour evaluations were assessed primarily according to RECIST 1.1 and iRECIST criteria, while patients with HCC were evaluated retrospectively using mRECIST. A total of 14 subjects are planned to be enrolled, and after the 14 subjects have completed the first cycle of dosing, pre-existing data from patients will be comprehensively analysed, and the trial will be considered to be discontinued if there is only 1 or less remission/response in subjects with pre-existing efficacy assessment and no significant benefit of VG161 over historical controls in other secondary endpoints of efficacy, such as the PFS rate, etc.; if, after review by the SRC, there is no significant benefit of VG161 in the treatment of hepatocellular carcinoma patients with clinical benefit, such as PFS rate (e.g. 4-month PFS rate) within a certain period of time is higher than the historical control data of regorafenib regorafenib, or there are 2 or more PRs, and the available safety and efficacy data show that the benefit of VG161 treatment outweighs the risk, the trial can be continued to the second phase, in which the tumour evaluations are mainly conducted in accordance with the RECIST 1.1 and mRECIST criteria, while referring to the iRECIST criteria (determination of disease progression is based on iCPD), and continue to add 16 subjects with advanced primary hepatocellular carcinoma to further investigate the efficacy and safety of VG161 in the treatment of hepatocellular carcinoma.

4.2 Study Procedure Design

4.2.1 Single-agent dose-escalation period

Subjects enter the screening period (-28days to -1 days) after signing the informed consent form; during the screening period, they will undergo a series of tests including performance status score (ECOG), vital signs, physical examination, hematology, blood biochemistry, urinalysis, herpes simplex virus type I antibody, 12-lead ECG and imaging (CT/MRI). According to the inclusion/exclusion criteria, eligible subjects will enter 5 dose groups in sequence.

After enrollment, subjects are given VG161 on Day 1. In different dose groups, the dose may be administered on only Day 1 or for 2-3 successive days from Day 1, and then subjects are observed until Day 28. The safety observation and pharmacokinetic sample collection of VG161 will be

conducted according to the protocol. In the accelerated titration or the dose-escalation phase of the 3+3 method, subject can continue the treatment with the IP at the same dose as that in Cycle 1 for a cumulative period of no more than 4 cycles, each cycle of 28 days only after subjects have completed the safety assessment in Cycle 1 for a total of 28 days and the safety has been confirmed, according to the investigator's determination and the principles of ethical medication and after discussion and decision by the SRC, until disease progression (iRECIST criteria, iCPD), no benefit from further treatment with the IP any longer in the opinion of the investigator, or intolerable toxicity, or voluntary request to withdrawal, or termination of the study for other reasons. If a subject achieves stable disease or partial response after 4 cycles, the SRC may decide whether to continue the treatment after discussion. If the subject's tumor lesion shrinks after intratumoral injection and cannot receive a further dose, the dose may be reduced based on the size of the injectable lesions as assessed by the investigator (refer to 6.2 Route and method of administration).

4.2.2 Monotherapy extension period

Subjects enter the screening period (-28 days to -1 days) after signing the informed consent form; during the screening period, they will undergo a series of tests including performance status score (ECOG), vital signs, physical examination, hematology, blood biochemistry, urinalysis, herpes simplex virus type I antibody, 12-lead ECG and imaging (CT/MRI). According to the inclusion/exclusion criteria, eligible subjects will sequentially enter the recommended dose group recommended in Period 1 to participate in the trial.

After the subjects are enrolled, they will receive VG161. According to the recommended dosage (including the number of continuous administrations), the administration day may be 2-3 consecutive days (one of the dose groups), and then subjects will be observed until the 28th day. The efficacy and safety of VG161 will be observed as per the protocol. Subjects will receive the investigational drug for up to 4 cycles of 28 days, or until disease progression (iRECIST criteria, iCPD) and no benefit from continuing treatment with the investigational drug in the opinion of the investigator, or intolerable toxicity, or voluntary request for withdrawal, or termination of the study for other reasons. If a subject achieves stable disease or partial response after 4 cycles, the SRC may decide whether to continue the treatment after discussion. If a subject cannot receive the dose due to shrinkage of tumor lesions after intratumoral injection, the dose is allowed to be reduced as appropriate based on the size of injectable lesions as assessed by the investigator (refer to 6.2 Route and method of administration).

Considering that immunotherapy may have pseudoprogression, the subject will be allowed to continue the investigational drug if the subject experiences disease progression (including new-onset lesions) initially, and does not experience rapid tumor progression with tolerable toxicity as assessed by the investigator, and can benefit from continuing the investigational drug in the opinion of the investigator. The tumor imaging will be performed again at least 4 weeks (not longer than 8 weeks) after the first evaluation of disease progression according to iRECIST. If disease progression is confirmed, the subject must discontinue the treatment.

See Section 8 (8 Study Procedures) for detailed study procedures.

4.3 Criteria for termination of trial

1) Presence of a significant safety risk to the IP in the opinion of regulatory authorities, ethics committees, the sponsors or the investigator.
2) The sponsor may terminate the study for any scientific, medical, or ethical reason, but must take full account of the rights, safety and well-being of the subjects in the group.
3) Other reasons for inappropriate continuation of the trial in the opinions the sponsor or the investigator.

5 Endpoints

5.1 Single-agent dose-escalation period

5.1.1 Primary endpoints

1) MTD/recommended dose;
2) Occurrence and frequency of DLT;
3) Occurrence and frequency of AEs, SAEs (according to NCI CTCAE 5.0).

5.1.2 Secondary endpoint

1) Pharmacokinetic parameters: Blood concentration of VG161 (copy number of specific VG161 DNA not present in wild-type HSV), PD-L1 blocking peptide and IL12 serum concentrations and at different time points after single and multiple doses;
2) Efficacy: Objective response rate (ORR), progression-free survival (PFS), duration of response (DOR) and one-year OS rate determined according to iRECIST and RECIST criteria;
3) Pharmacodynamic and immunology parameters: Peripheral blood lymphocyte subsets (CD3+, CD4+, CD8+, CD4+/CD8+ ratio, CD19+, CD16+CD56+ (NK) cells), plasma cytokines (IL-15, IL-6, TNF-a, IFN-γ), peripheral blood T cell immunity and other immune-related indicators (PD-L1, PD-1, CD69, CD8+Ki67high) and serum VG161 anti-drug antibodies.

5.2 Monotherapy extension period

5.2.1 Primary endpoint(s)

1) Objective response rate (ORR);

5.2.2 Secondary endpoints

1) Progression-free survival (PFS) and PFS rates at specific time points (e.g., 4 months, etc.);

2) One-year survival rate (OS rate) and overall survival (OS);

3) Duration of response (DOR);

4) Safety evaluation, including adverse events, laboratory parameters and other safety data (electrocardiogram, vital signs, etc.);

5) Pharmacodynamic indicators: peripheral blood lymphocyte subsets (CD3+, CD4+, CD8+, CD4+/CD8+ ratio, CD19+, CD16+CD56+ (NK) cells), plasma cytokines (IL-15, IL-6, TNF-a, IFN-γ), peripheral blood T cell immunity and other immune-related indicators (PD-L1, PD-1, CD69, CD8+Ki67high).

The efficacy parameters for the dose-escalation period of this trial, as well as the first period of the monotherapy expansion period of the Simon's two-period design, will be evaluated according to the iRECIST and RECIST1.1 criteria, respectively, and HCC patients will be evaluated retrospectively according to the mRECIST criteria. The efficacy parameters for the second period of the Simon's two-period design to monotherapy expansion will be evaluated according to the RECIST 1.1 and mRECIST criteria, respectively, with reference to the iRECIST criteria (disease progression will be determined by the iCPD).

6 Treatment

6.1 Investigational Product

6.1.1 Overview of investigational product

Product Code: VG161 (recombinant human IL12/15-PDL1B oncolytic herpes simplex virus type I injection (Vero cells));
Batch no. (tentative): 20200702 (titer: 5.93 x 10^7 PFU/ml, expiry date:20220716);
Strength: 1.0 ml/vial (vial);
Description: slightly opalescent liquid;
Concentration: ≥1×10^7 PFU/ml (as indicated by the manufacturer);
Formulation composition: VG161, 50 mM tromethamine, hydrochloric acid, 150 mM sodium chloride, 5% glycerol;
Shelf life: 2 years;
Storage: below -60°C;
Manufacturer: GeneSail Biotech (Shanghai) Co., Ltd.;
Usage: intratumoral injection.

6.1.2 Preparation and Dispensing

The sponsor is responsible for cold chain shipment (below -60°C) of the investigational drug to the study site, the investigator/pharmacist signs an acknowledgment of receipt, and stores the drug below -60°C.
The IP will only be used for this study and be managed by a person authorized by the investigator. The investigator/pharmacist in charge of drug management is responsible for storing, dispensing, and returning the IP according to the study procedures and accurately recording the process of receiving, dispensing, and returning the IP. Unused or expired drug will be returned and destroyed by the sponsor.

6.2 Dosage and administration

After removal, VG161 stored below -60°C is thawed at room temperature and then diluted with normal saline according to tumor size before use(if applicable). This product must not be frozen and thawed repeatedly. Keep at room temperature for no more than 2 hours.

The location of the tumour is shown by a diagnostic ultrasound/CT machine. If ultrasound guidance is used, CDFI shows the blood supply within the tumour as well as the large blood vessels surrounding the tumour, and the presence or absence of necrotic areas of the tumour, etc., is shown on the ultrasound image map by injecting 2.4 ml of Sono Vue intravenously.The use of CT instrumentation is carried out in accordance with the regulations of the study centre. Under ultrasound or CT guidance, a PTC needle was used to puncture into the active tumour tissue avoiding blood vessels, and the appropriate volume of VG161 was injected according to the size of the tumour in a manner of injecting while backing out of the needle to allow for uniform distribution of the drug, and after the drug injection was completed, the drug was rinsed with approximately 0.5 ml of normal saline.

If necessary, local anesthetic may be used at the injection site, but must not be injected directly into the lesion. Try to keep off the lesion or injection position close to the large blood vessels or important nerves.

Refer to the Mylab-Twice ultrasonic diagnostic instrument (Esaote, Italy), the probe is CA541, and the frequency is 2.0-4.0 MHz; The puncture needle is a 18G PTC needle (HAKKO, Japan); the ultrasound contrast agent is Sono Vue (Italy).

Superficial lesions can be injected visually. VG161 can be injected uniformly into the lesions through intratumoural multi-point injection by removing the needle without withdrawing from the lesion and changing the direction of needle insertion. If necessary, multiple injections may also be given to achieve complete and uniform dispersion of the liquid medicine.

When removing the needle, withdraw the lesion slowly to avoid leakage at the insertion point.

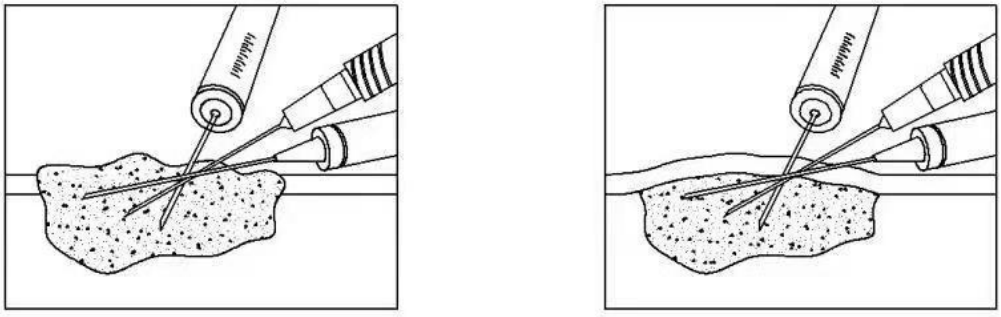


Figure 15: Schematic diagram of intratumoral injection

It is recommended that the investigator determine the dose volume based on the size of the injected lesion (see Table 4 below). This recommended injection volume is for the investigator's reference. The investigator can determine the final injection volume based on the actual lesion condition. If the size of the lesion does not meet the criteria for injection, but the investigator believes that the lesion can be injected based on its location and texture, the subject is allowed to be included.

Table 4: Determination of dose volume based on size of injected lesion

Lesion size (sum of the longest diameter) (Lymph node lesion short diameter)	Recommended injection volume (Can be diluted with normal saline)
＞5cm	4ml
＞2.5-5cm	2ml
＞1.5cm-2.5cm	1ml

If concurrently existing in a subject, multiple lesions may also be selected for injection. For example, if the dose group needs to inject 2 ml of the investigational drug, a single lesion of > 2.5 cm can be chosen, or two lesions of > 1.5 to 2.5 cm can be injected for 1 ml each. However, the injection for multiple lesions may increase subjects' discomfort, especially when the high-dose group needs many consecutive days of injection, so a single larger lesion for injection of the investigational drug is preferentially recommended. When selecting one or several lesions in

multiple injectable lesions for injection, in the case of multiple consecutive injections, the injected lesion should be selected first for continuous injection, and when it is difficult to perform further intratumoral injection, other injectable lesions can be selected for injection. Please refer to 6.3.1 and 6.3.2 for the principles of dose adjustment during the dose-escalation phase.During the dose-therapy expansion phase, when there are no other injectable lesions after 1 injection in the cycle, subsequent treatment may be delayed for a maximum period of time not exceeding the current cycle, as determined at the discretion of the investigator. If it is necessary to reduce or suspend the dose of VG161 intratumoural injection because the lesion has shrunk or disappeared, the dose may be reduced or the administration may be suspended depending on the actual lesion.

6.3 Handling of expected toxicity and dose titration principles

6.3.1 Dose titration during the DLT observation period

During the DLT observation period, any dose titration is not allowed for VG161. For subjects who require more than 1 injection, if it is difficult to perform further intratumoral injection at the injection site after 1injection, and there are no other injectable lesions, subsequent treatment can be performed by injection every other day at the discretion of the investigator. If fever occurs, it is recommended to give physical cooling and non-hormonal antipyretic analgesics as well as blood routine examination and blood biochemistry of liver and kidney function. If hematological examination results are normal and no infection is found,liver puncture is not contraindicated and CRS manifestation doesn't occur, Body temperature is below 38.5℃ before administration and the interval between two doses does not exceed 60h. After assessment by the investigator, the next dose preset in this cycle can be continued.If a DLT occurs, the subject should be actively managed and should discontinue the study treatment unless the event resolves to ≤ Grade 1 or baseline within 7 days and the investigator believes that it is in the best interest of the subject to continue the treatment. In such case, the study treatment may be resumed after discussion with the sponsor.

6.3.2 Dose titration beyond the DLT observation period

If a subject experiences ≥ Grade 3 toxicity outside the DLT observation period and the investigator may withhold the administration of VG161 if he/she believes that there is a safety risk. If a dose delay exceeds the scheduled dose by > 3 weeks, the treatment will be discontinued. The treatment may be delayed for 8 weeks if the subject experiences an immune-related adverse event (irAE) in the opinion of the investigator.

6.3.3 Handling of expected toxicity

6.3.3.1 Oncolytic virus related toxicity

According to the safety data from clinical trials of oncolytic viruses with similar drugs HSV-1 as carriers, the main toxic reactions after oncolytic virus injection include inflammation, redness, fever, fatigue, chills and other flu-like symptoms at the injection site, most of which are transient and healed without treatment. Notably, the incidence and severity of local injection site reactions and fever in HSV-1 antibody-negative subjects are higher than those in HSV-1 antibody-positive subjects. Since this study is an early human study of VG161, only HSV-1 antibody-positive

subjects will be included from a safety perspective.

1) Flu-like symptoms

The most common systemic symptoms are flu-like symptoms such as fever, fatigue and chills. If the subject has Grade 1 to 2 fever, only physical cooling is recommended，using non-hormonal antipyretic analgesics sparingly. If the subject has Grade ≥3 fever, the investigator may give antipyretic analgesics. Corticosteroids are contraindicated for subjects without irAE manifestations except flu-like symptoms. If they experience persistent hyperthermia, hypoxia or decreased blood pressure, subjects should be alert to cytokine release syndrome (CRS). See Section 6.3.3.3 for details.

2) Injection site reaction

According to the oncolytic properties of the IP and the method of administration of local injection, as well as the local injection reaction observed in the preclinical toxicology experiment of VG161, injection site reactions, such as pain, erythema, swelling, and increased skin temperature, necrosis, and ulcers, may occur with superficial lesion injection. Tumor tissue necrosis or ulcers attributable to superficial lesion injection and even cellulitis and systemic bacterial infection in severe cases, have been reported in the clinical study on similar drug T-Vec. It is advised to give symptomatic treatment if any reaction occurs at the injection site during the test. If necrosis or ulcers occur, careful care should be taken for the wound while infection prevention measures should be taken. In case of concurrent infection, local or systemic anti-infective treatment should be given, or even the surgical drainage is needed.

3) HSV-1 infection

HSV-1 infection may manifest as herpes labialis, herpetic keratitis, herpetic dermatitis and lead to viral encephalitis in severe cases. Therefore, if a subject develops a suspected herpes-like lesion during the study, safety precautions should be taken immediately (see Section 6.4 for details). If he/she believes that the subject has manifestations of HSV infection and faces a safety risk, the investigator may withhold VG161 and give acyclovir for treatment.

6.3.3.2 irAE

Although the concentrations of IL12, IL15/RA, and PD-L1 blocking peptides released into the blood are expected to be very low, it is still necessary to closely observe the occurrence of irAEs. According to the clinical trial report of PD-1/PD-L1 monoclonal antibody, irAEs include the toxicity of multiple systems, such as skin, gastrointestinal tract, liver, endocrine, lung and musculoskeletal systems;. However, most irAEs were mild to moderate in severity, serious and fatal irAEs have been reported, such as immune-mediated pneumonitis or hepatitis.

An irAE usually requires treatment with corticosteroids. In general, oral or intravenous corticosteroids (such as prednisone) can be used when irAE is ≥ Grade 2. Considering the synergistic anti-tumor immune effect of VG161, it is recommended to use corticosteroids with caution. Mild skin toxicity may be treated with topical corticosteroids or oral antihistamines. If endocrine-related toxicity occurs and related endocrine gland dysfunction exists, exogenous hormone replacement therapy (such as levothyroxine, hydrocortisone, etc.) may be given.

After initiation of corticosteroid therapy, the dose is usually tapered after toxicity resolves to ≤ Grade 1. It will take at least 4 to 6 weeks to taper the dose until discontinuation or the resumption of the investigation drug may be considered when prednisone is ≤ 10 mg/day.

If an irAE occurs During the study, the IP may be withheld or discontinued according to the table below (Table 5).

Table 5: irAE classification

Classification of AEs	Name of AE	CTCAE Grade	Action taken against the IP
Skin toxicity	Maculopapular rash/dermatitis	Grade 3	Withhold dose until toxicity resolved to ≤ Grade 1 or baseline
		Grade 4	Permanently withdrawn
Toxic gastroenteritis	Colitis	Grade 2	Withhold until toxicity resolved to ≤ Grade 1 and the dose of corticosteroids is reduced to ≤ 10 mg/day of prednisone
		Grade 3	For the first occurrence, withhold until toxicity resolved to ≤ Grade 1 and the dose of corticosteroids is reduced to ≤ 10 mg/day of prednisone; continue the treatment until recurrence and then discontinue
		Grade 4	Permanently withdrawn
Hepatotoxicity	Hepatitis (elevated ALT, AST or total bilirubin)	Grade 2	Withhold until toxicity resolved to ≤ Grade 1 and the dose of corticosteroids is reduced to ≤ 10 mg/day of prednisone
		≥ Grade 3	Permanently withdrawn
Endocrine related toxicity	Hypophysitis	≥ Grade 2	Withhold until adequate hormone replacement is provided
	Thyroid function decreased	≥ Grade 3	Withhold until toxicity resolved to ≤ Grade 2
	Hyperthyroidism	≥ Grade 3	Withhold until toxicity resolved to ≤ Grade 1
	Type 1 diabetes mellitus	≥ Grade 3	Withhold until toxicity resolved to ≤ Grade 1
		Hyperglycemia of any grade with diabetic ketoacidosis	Withhold until complete recovery from diabetic ketoacidosis
Pulmonary toxicity	Pulmonitis	Grade 1-2	Withhold until lung imaging results indicate complete recovery
		≥ Grade 3	Permanently withdrawn
Rheumatic disease	Arthritis	Grade 2	Withhold until toxicity resolved to ≤ Grade 1 and the dose of corticosteroids is reduced to ≤ 10 mg/day of prednisone
		Grade 3	Withhold until toxicity resolved to ≤ Grade 1 and the dose of corticosteroids is reduced to ≤ 10 mg/day of prednisone; Discontinue if symptoms do not improve after 4-6 weeks
Cardiovascular system toxicity	Myocarditis/pericarditis	Grade 3	Withhold until toxicity resolved to ≤ Grade 1

		Grade 4	Permanently withdrawn
Hematological Toxicity	Anemia	Grade 3	Withhold and resume if resolved to ≤ Grade 2 with corticosteroids
		Grade 4	Permanently withdrawn
	Platelets decreased	Grade 3	Withhold until resolved to ≤ Grade 1; Discontinue if toxicity does not resolve after treatment with corticosteroid
		Grade 4	Withhold until resolved to ≤ Grade 1; Discontinue if toxicity does not resolve after treatment with corticosteroid
Nephrotoxicity	Nephritis	Grade 2	Withhold until toxicity resolved to ≤ Grade 1
		Grade 3	Withhold and if toxicity resolved to ≤ Grade 1 and the investigator believes that the subject may probably benefit from further treatment, the IP may be resumed
		Grade 4	Permanently withdrawn
Nervous System Toxicities	Encephalopathy/leukoencephalopathy/posterior reversible encephalopathy syndrome	Grade 1-2	Withhold until complete recovery from toxicity
		≥ Grade 3	Permanently withdrawn
	Peripheral motor and sensory neuropathy	Grade 2	Withhold until toxicity resolved to ≤ Grade 1
		≥ Grade 3	Permanently withdrawn
Ocular toxicity	Uveitis	Grade 2	Withhold until toxicity resolved to ≤ Grade 1
		Grade 3	Discontinue; if the subject recovered from the toxicity after treatment and the investigator believes that the subject can probably benefit from further treatment, the IP may be resumed
		Grade 4	Permanently withdrawn
	Episcleritis	Grade 2	Withhold until toxicity resolved to ≤ Grade 1
		Grade 3	Discontinue; if the subject recovered from the toxicity after treatment and the investigator believes that the subject can probably benefit from further treatment, the IP may be resumed
		Grade 4	Permanently withdrawn

6.3.3.3 Cytokine release syndrome

Cytokine Release Syndrome (CRS) is generally a serious excessive immune response attributable to the explosive secretion of large amounts of cytokines by immune cells during tumor treatment, resulting in non-specific inflammation. Systemic symptoms induced by CRS include fever, nausea, chills, hypotension, hypoxia, headache and skin rashes. In severe cases, it can cause damage to multiple organs. If the subject has the above symptoms, the investigator should promptly detect cytokine (such as IL-6). If subjects have persistent mild CRS persist or moderate to severe CRS,

Tocilizumab (IL-6 monoclonal antibody) and/or corticosteroids should be given in time. Considering the synergistic anti-tumor immune effect of VG161, it is recommended to use corticosteroids with caution. In addition, corresponding supportive treatment, such as oxygen inhalation, antipyretic analgesics, vasopressors, should be given.

6.3.3.4 Tumor lysis syndrome

Tumor lysis syndrome is a condition caused by the rapid destruction of tumor cells and the release of a large number of cell contents into blood vessels during tumor treatment, which is manifested by hyperuricemia, hyperphosphatemia, hypocalcemia, hyperkalemia, acute uric acid nephropathy and other symptoms. If not detected promptly and treated correctly, it can cause kidney failure, arrhythmia and convulsions, thus leading to death. During the treatment process, it is necessary to be alert to tumor lysis syndrome. For high-risk patients as determined by the investigator, hydration and alkalization can be given while injecting the IP. If a subject is suspected with tumor lysis syndrome during treatment, his/her urine output and laboratory parameters should be monitored promptly and hydration, alkalization and allopurinol should be given.

6.3.3.5 Potential changes in hematology and toxicity

In the previous chronic toxicity test of VG161 in monkeys, different levels of reduction in RBC (red blood cells), HGB (hemoglobin) and HCT (hematocrit) and prolonged APTT (activated partial thromboplastin time) were found in each dose group (high, medium and low), but no related toxic reactions and pathological changes were observed. In phase I clinical trials, attention should be paid to the changes in the above parameters. If major changes or related clinical symptoms present, preventive and symptomatic treatment measures should be actively taken.

6.4 Biosafety precautions

HSV-1 type has become one of the most widely used gene vectors because of its wide host range, low toxicity, large capacity, non-integration and other characteristics. Now, it has been largely transformed into various types of oncolytic viruses for cancer treatment and research and its safety has also been widely proven.

Accidental exposure may lead to the spread of VG161 and HSV-1 infection. Therefore, the treatment personnel should pay attention to their own protection during operation, such as wearing masks, hats and protective goggles in order to prevent the liquid from foaming or splashing. If the hands, face and other skin and objects are accidentally contaminated, immediately wipe with 75% ethanol and then rinse with water. If the liquid splashes into the mucous membranes of the eyes, mouth, nose and other parts, immediately rinse with water repeatedly. Empty vials, used dressings, gloves and cleaning materials after dispensing should not be discarded without approval and should be disposed strictly as medical hazardous waste, so the safety measures should be carefully taken during the disposal.

Subjects should try to avoid contact with the injection site or its dressing since doing so may result in inadvertent transfer of VG161 to other areas of the body. Close contacts (caregivers, family members and patients in the same ward, etc.), pregnant women and newborns should avoid direct

contact with the injection site, dressings or body fluids of the treated subject.

Subjects who develop suspected herpes-like lesions should first follow standard hygiene measures to prevent the spread of the virus and contact their supervising physician for evaluation and treatment as soon as possible.

Herpes simplex virus is sensitive to acyclovir, but acyclovir or other antiviral drugs may interfere with the efficacy of VG161. Therefore, before starting treatment of herpes infection with antiviral drugs, the benefits and risks of VG161 therapy should be considered comprehensively.

6.5 Concomitant drugs and therapies

6.5.1 Prohibited foods, medications and treatments

During the trial, any anti-tumor therapy (including cytotoxic chemotherapy, targeted drugs, radiotherapy, endocrine therapy and traditional Chinese medicine with anti-tumor indications) and other clinical IP are prohibited.

Except for corticosteroids that have to be used due to toxic reactions, other immunosuppressive agents and drugs that affect the immune system (such as interferon, thymosin, etc.) are prohibited. Long-term use of corticosteroids (＞10 mg/day of prednisone or equivalent) is prohibited.

Except for those for treatment of toxicity, all anti-herpes simplex virus drugs are prohibited during the study, including but not limited to: acyclovir, valaciclovir, valacyclovir, penciclovir, famciclovir, ganciclovir, and cidofovir, etc. If patients with inactive chronic hepatitis B have viral reactivation and need to use Anti-HBV virus drugs, or other situations require use of antiviral drugs, it is recommended that the investigator discusses with the sponsor to evaluate the risks and benefits of subjects continuing to participate in this study.

6.5.2 Permitted drugs and therapies

The following medications are allowed to be used as appropriate during the study:

(1) Long-term medication required for concomitant diseases (such as hypertension, diabetes, etc.);
(2) Hormone replacement therapy or oral contraceptives for any purpose other than anti-tumor therapy;
(3) If the subject is positive for HBV-DNA, the subject should receive treatment with entecavir, or tenofovir disoproxil fumarate, or tenofovir alafenamide fumarate, or lamivudine according to 'Guidelines for the prevention and treatment of chronic hepatitis B' (2019 Edition);
(4) Symptomatic and supportive treatment for drug toxicity or control tumor symptoms. Patients with bone metastases are allowed to receive bisphosphonate therapy;
(5) Other concomitant drugs and treatments which are used as determined by the investigator on the premise of protecting the interests and safety of subjects;
(6) All Concomitant drugs and therapies within 28 days before the first dose and within 28 days after the last dose (or until the subject initiates a new anti-tumor therapy) should be recorded on the case report form (CRF) together with the reason for the medication.

7 Sample Collection and bioanalysis

All sample collection time points and time windows described in this section are summarized in attached Appendix Table 2.

7.1 Pharmacokinetics/virus excretion and serum antibodies (For Period 1. Single-agent dose-escalation period only)

Blood samples, urine/fecal samples, saliva (oral) swabs, and injection site swabs for pharmacokinetics/viral excretion and antibody detection will be collected from all subjects during the single-agent dose-escalation period. The pharmacokinetic test indicators are blood concentration of VG161 (copy number of specific VG161 DNA not present in wild-type HSV), PD-L1 blocking polypeptide plasma concentrations and IL12 serum concentrations and serum antibodies (anti-HSV-1 IgG, anti-drug antibody ADA and neutralizing antibody NAb) at different time points after dosing in Cycle 1. Samples will be tested and analyzed in a central laboratory. The procedures for sample handling, storage and shipment are detailed in the *Laboratory Manual*.

7.1.1 Blood sampling

A total of 4 ml of venous blood is collected at each time point (except for serum antibody* detection).

The collection time points vary by dose group and are detailed in the following table:

Table 6: PK blood sampling time points

Dosing regimen	PK blood sampling time points
1.0×10^8 PFU 1-day dosing	D1 (day of dosing): pre-dose***, 1h, 2h, 6h, 12h post-dose; D2: 24h after dosing; D3: 48 h after dosing; D5: 96 h after dosing; D8: 168 h after dosing; D15、D22、D28
1.0×10^8 PFU 2-day** dosing	Pre-dose on Day 1 (D1), pre-dose on Day 2 (D2), pre-dose and post-dose 1h、2h、6h、12h、24h (D3)、48h (D4)、96h (D6)、168h (D9)、D15、D22 and D28
1.0×10^8、 1.3×10^8、 1.7×10^8PFU 3-day** dosing	Pre-dose on Day 1 (D1), pre-dose on Day 3 (D3), pre-dose and post-dose 1h、2h、6h、12h、24h (D4)、48h (D5)、96h (D7)、168h (D10)、D15、D22 and D28

Note(s):

*Serum antibody detection: For the 1-day dosing group, additional 2 ml of venous blood is to be collected for anti-HSV-1 IgG(ADA) baseline detection and 5 ml of venous blood for anti-PD-L1 blocking polypeptide (ADA) and anti-HSV-1 IgG(Nab) baseline detection within the first 2 days (D-2) of dosing; for the 2-3 day dosing group, additional 2 ml of venous blood is to be collected for anti-HSV-1 IgG(ADA) baseline detection and 5 ml of venous blood for anti-PD-L1 blocking polypeptide (ADA) and anti-HSV-1 IgG(NAb) baseline detection before

dosing on the first day of dosing (D1). For 1-3 d dosing group, only 2ml of venous blood is collected 12h after dosing for anti-HSV-1 IgG(ADA) detection on the day of intensive blood sampling (5 time points); additional 7 ml of venous blood will also be collected for the detection of anti-HSV-1 IgG(ADA), anti-PD-L1 blocking polypeptide (ADA) and anti-HSV-1 IgG(NAb) on each single blood collection day (24 h, 48 h, 96 h, 168 h after administration and D15, D22,D28).** means the actual number of injection days. If the drug is administered every other day between the second and/or the third, it is actually Day 3 and Day 4/5 of the course of treatment. ***Pre-dose refers to within -2 hours before dosing on the day of dosing, the same below.

7.1.2 Urine and fecal sample collection

Urine sample collection: A total of 10 ml of urine sample is collected from the subject at the specified time point (urinating before dosing or the first urinating on the morning of the same day). Fecal sample collection: About one centrifuge tube of fecal sample should be collected as pre-dose sample from any defecation of the subject within 2 days before dosing; about one centrifuge tube of fecal sample should be collected as post-dose sample from any defecation of the subject within 48 hours after completion of the protocol-specified dose. Any subject who fails to provide the sample due to constipation should be recorded.

Table 7: Time points for collection of urine and fecal samples

Dosing regimen	Time points for collection of urine and fecal samples
1.0×10^8 PFU 1-day dosing	Urine sample before dosing on the day of dosing (D1) and on the morning of D2, D15, D22 and D28; Fecal sample within the first 2 days of dosing (D-2), each fecal sample up to 48 hours after dosing
1.0×10^8 PFU 2-day** dosing	Urine samples before dosing on Day 1 (D1), before dosing on Day 2 (D2) and on the morning of D3, D15, D22 and D28 Fecal sample before the first dose (D1), and each fecal sample up to 48 hours after the second dose
1.0×10^8 、 1.3×10^8、 1.7×10^8PFU 3-day** dosing	Urine samples before dosing on Day 1 (D1), before dosing on Day 3 (D3) and on the morning of D4, D15, D22 and D28 Fecal sample before the first dose (D1), and each fecal sample up to 48 h after the last dose

Note:** means the actual number of injection days. If the drug is administered every other day between the second and/or the third, it is actually Day 3 or Day 4/5 of the course of treatment.

7.1.3 Saliva swab sample

Saliva (oral) swab samples will be collected from subjects before dosing on Day 1 (C1D1), before dosing every day during consecutive dosing period (only for 2-3 day dosing group), and 0.5 h, 1 h, 4 h, 8 h, 24 h after the last dose, as well as on C1D15, C1D28 (or before dosing on C2D1).

7.1.4 Swab of injection site

Swabbing samples at injection site should be collected only from subjects who have received injection into superficial lesions. Swabbing samples at injection site will be collected from subjects before dosing on Day 1 of Cycle 1(C1D1), before the last dose and 0.5 h, 1 h, 4 h, 8 h, 24 h after the end of dosing period.

7.2 Pharmacodynamic and biomarker studies (for Period 1 and Period 2)

7.2.1 Blood sampling and testing

Table 8: Pharmacodynamics and biomarker blood sampling time points

(Applicable to Period 1 Cycle 1)

Dosing regimen*	Pharmacodynamic and biomarker blood sampling time points
1-day dosing group	Within the first 2 days of dosing (D-2), 24 h after dosing (D2), D15 and D28
2-5 day dosing group	Pre-dose on Day 1 (D1), before the last dose, 24 h after the last dose, D15 and D28

Note: * means the actual number of injection days. If the drug is administered every other day between the secondand/or the thirdit is actually Day 3 or Day 4/5 of the course of treatment.

Table 9: Pharmacodynamics and Biomarker Blood Sampling Time Points
(Applicable to each cycle in Period 2 and Period 1 Cycle 2 and subsequent cycles)

Dosing regimen*	Pharmacodynamic and biomarker blood sampling time points
1-5 day dosing group (1 of the groups)	Pre-dose on Day 1 (D1) of each cycle, 24 h after the last dose of each cycle, D15 and D28

Note: * refers to the number of actual injection days.

1) Lymphocyte subsets in peripheral blood

Samples should be collected from all subjects.

The detection indicators of peripheral blood lymphocyte subsets are CD3+, CD4+, CD8+, CD4+/CD8+ ratio, CD19+ and CD16+CD56+ (NK) cells. The testing is conducted by the laboratory at the site and the sample collection and processing procedures will be subject to the provision of the site.

2) Plasma cytokines

A total of 2ml of venous blood should be collected from all subjects at each time point.

The detection indicators of plasma cytokines are IL-15, IL-6, TNF-a, IFN-γ. Samples will be tested and analyzed in a central laboratory. The procedures for sample handling, storage and shipment are detailed in the *Laboratory Manual*.

3) T cell immune related indicators in peripheral blood

A total of 4 ml of venous blood should be collected from all subjects at each time point.

Peripheral blood T cell immunity and other immune-related indicators: the specific detection indicators are PD-L1, PD-1, CD69 and CD8+Ki67high. Samples will be tested and analyzed in a central laboratory. The procedures for sample handling, storage and shipment are detailed in the *Laboratory Manual*.

8 Study procedures

8.1 Observations

Table 10: Clinical observation indicators

Observations	**Description**
Demographic data	Age, gender and ethnicity, etc.
Medical history and medication history	History of systemic diseases, medication history and all past anti-tumor treatments
Vital Signs	Body temperature, respiration, pulse/heart rate and blood pressure
Physical measurement	Height (at screening period only) and weight
Physical examination	The assessment is performed by organ and system. A comprehensive physical examination includes: general condition, head and neck, lymph nodes, skin, chest,

	abdomen, musculoskeletal system (including limbs and spine); a brief physical examination includes, but is not limited to, general condition, mouth and lips, skin at the injection site and any abnormal signs of interest in the opinion of the investigator
ECOG performance status score	As per ECOG performance status score (see Appendix 3)
Infectious disease markers	Hepatitis B surface antigen (HBsAg), hepatitis B surface antibody (HBsAb), hepatitis B core antibody (HBcAb), hepatitis C antibody (Anti-HCV), hepatitis B virus DNA (HBV-DNA), hepatitis C virus RNA (HCV-RNA), human immunodeficiency virus antibody (HIV) and treponema pallidum antibody (RPR or TRUST)
Herpes simplex type I antibody	HSV-1 IgG、HSV-1 IgM
Hematology	White blood cell count (WBC), red blood cell count (RBC), hemoglobin (Hb), platelet count (PLT), neutrophil count (ANC) and lymphocyte count (LYM)
Urinalysis	Protein, glucose, white blood cells, red blood cells and occult blood
Coagulation function	Prothrombin time (PT), activated partial thromboplastin time (APTT), international normalized ratio (INR) and fibrinogen (FIB)
Blood biochemistry (*Liver function*)	Serum alanine aminotransferase (ALT), aspartate aminotransferase (AST), albumin (ALB), total bilirubin (TBIL), direct bilirubin (DBIL), alkaline phosphatase (ALP) and gamma-glutamyltransferase (GGT)
(*Renal function*)	Creatinine (CREA), urea/urea nitrogen (UREA/BUN) and uric acid (UA)
(*Electrolytes*)	Potassium (K), sodium (Na), chloride (Cl), calcium (Ca), magnesium (Mg) and phosphorus (P)
	Fasting blood glucose (GLU)
	Amylase (AMS) and lipase (LPS)
Thyroid function	Free triiodothyronine (FT3), free thyroxine (FT4) and thyroid-stimulating hormone (TSH)
Pregnancy test	Blood HCG pregnancy test for women of childbearing potential
12-lead ECG	Heart rate, PR interval, QTc interval and ECG abnormalities
Echocardiography	Left ventricular ejection fraction (LVEF)
Tumor assessments	CT/MRI, etc. as assessed according to iRECIST (see Appendix 5) and RECIST 1.1

All laboratory tests will be performed in a laboratory that the site has accredited. The normal range for all laboratory tests and updates during the trial should be recorded on the appropriate pages of the CRF.

8.2 Study procedures

8.2.1 Screening/baseline period (Day -28 to Day -1)

Unless otherwise specified, the screening assessments must be completed within 28 days prior to initiation of treatment with the IP. To protect the rights and interests of subjects, the relevant examinations that have been completed before signing the informed consent form may not be repeated if they meet the requirements of the protocol and within the specified time window. The specific screening procedures and assessments are shown as follows:

A subject who does not meet the inclusion or exclusion criteria for the study or does not complete

all screening tests since 28 days after signing the ICF will be considered a screening failure. Screening failed subjects can be re-screened after appropriate treatment or observation at the investigator's discretion, and re-screened subjects will be assigned a new screening number and sign a new ICF. Each subject can be rescreened up to once.

1) Obtain the written informed consent;
2) Demographic data;
3) Collection of medical and medication history;
4) Vital signs (Day -7 to Day -1);
5) Height and weight (Day -7 to Day -1)
6) Complete physical examination (Day -7 to Day -1);
7) ECOG score (Day -7 to Day -1);
8) Hematology, urinalysis, coagulation function, biochemistry and thyroid function (Day -7 to Day -1);
9) Markers of infectious diseases(Day -7 to Day -1);
10) Herpes simplex type I antibody
11) Pregnancy test for women of childbearing potential (Day -7 to Day -1);
12) 12-lead ECG(Day -7 to Day -1);
13) Echocardiogram;
14) Baseline tumour assessment (CT/MRI, etc.): if a subject has had a CT/MRI within 28 days prior to initiation of the drug and can be assessed according to RECIST criteria, the results of this imaging may be used as a baseline tumour assessment during the screening period;
15) Pre-dose baseline blood samples are collected. The specific sampling time points and requirements are detailed in Section 7 (7 Sample collection and testing) and Appendix Table 2.
16) Record adverse events after signing the ICF;
17) Concomitant drugs and therapies within 28 days prior to the first dose should be recorded.

8.2.2 Treatment cycle

A. Single-agent dose-escalation period (period 1)

a. Cycle 1 (C1D1-C1D28)

C1D1-C1D7 (single day dosing group)

1.When the IP VG161 is administered via single intratumoral injection on C1D1, vital signs are monitored before dosing (-2) and 2h±15min, 4h±15min, 24h(±1h) and 48h(±1h) after dosing.

2. Collect blood, urine, fecal samples, etc. The specific sampling time points and requirements are detailed in Section 7 (7 Sample collection and testing) and Appendix Table 2.

3. Record adverse events, concomitant drugs and therapies.

C1D1-C1D7 (multi-day dosing group)

1. According to the protocol, the dose group is given the IP VG161 intratumorally once a day for 2 to 3 days (from C1D1 to C1D3). Vital signs are monitored daily before dosing (within-2h) and 2 h (± 15 min) and 4 h (± 15 min) after dosing.

2. Vital signs should be monitored at 24 h (± 1 h) and 48 h (± 1 h) after the last dose.
3. Collect blood, urine, fecal samples, etc. The specific sampling time points and requirements are detailed in Section 7 (7 Sample collection and testing) and Appedix Table 2.
4. Record adverse events, concomitant drugs and therapies.

C1D8-C1D28

1. The following safety assessments will be performed each cycle(D8, D15, D22), allowing for a time window of ±1 day.

1) Vital signs;
2) Brief physical examination;
3) Laboratory test: hematology, urinalysis and blood biochemistry;
4) 12-lead ECG.

2. The following assessments will be performed at the end of Cycle 1 (C1D28±1, but before dosing in Cycle 2):

1) Vital signs;
2) ECOG score;
3) Weight
4) Brief physical examination;
5) Laboratory test: hematology, urinalysis, blood biochemistry, coagulation function and thyroid function;
6) 12-lead ECG;

3. Collect blood and urine samples, etc. The specific sampling time points and requirements are detailed in Section 7 (Sample collection and testing) and Appendix Table 2.
4. Tumor assessment: The tumor imaging examination will be performed initially at the end of the first cycle (C1D28), The time window for examination is ±3 days.
5. If the subject has an abnormal 12-lead ECG, myocardial enzyme test and echocardiography may be conducted as deemed necessary by the investigator. In addition, the investigator may add unscheduled tests as clinically indicated.
6. Record adverse events, concomitant drugs and therapies.

b. Cycle 2 (C2D1-C2D28) and subsequent cycles

After subjects in have completed the safety assessment of the first cycle for a total of 28 days and the safety has been confirmed, according to the investigator's judgment and the principles of ethical medication and after discussion and decision by the SRC, subjects can continue to receive the IP at the same dose as that in the first cycle for a cumulative period of no more than 4 cycles of 28 days, until disease progression (iRECIST criteria, iCPD) and no benefit from continuing treatment with the IP in the opinion of the investigator, or intolerable toxicity, or termination of the study for other reasons. If a subject achieves stable disease or partial response after 4 cycles, the SRC may decide whether to continue the treatment after discussion. Safety assessment visits and tumor assessments will be performed at the time points specified below.

1. Starting from Cycle 2, each cycle consists of 28 days. According to the administration method

used in Cycle 1, subjects will receive an intratumoral injection of VG161 on the first 1 to 3 days of each cycle (C2D1-C2D3, C3D1-C3D3…). A time window of ±1 day is allowed for the first day of Cycle 2 and a time window of ±3 days is allowed for the third and subsequent cycles.

Vital signs are monitored and recorded daily at before dosing (within2h) and 2 hours (±15 min) after dosing in Cycle 2 of injection. If a subject has not shown abnormality in vital signs during the dosing period, his/her vital signs may be tested at the discretion of the investigator in subsequent cycles.

2. The following safety visits will be performed starting from Cycle 2 and at the end of each cycle (C2D28, C3D28…); a time window of ±3 days is allowed:

1) Vital signs;
2) ECOG score
3) Weight
4) Brief physical examination;
5) Laboratory test: hematology, urinalysis and blood biochemistry;
6) Laboratory tests: coagulation function, thyroid function, at the end of every two cycles (C3D28, C5D28...);
7) 12-lead ECG.

3. Collect blood samples. The specific sampling time points and requirements are detailed in Section 7.2 (7.2 Pharmacodynamic and biomarker studies) and attached Table 2.

4. Tumor assessment: Tumor imaging examination will be performed at the end of the first cycle (C1D28), and thereafter performed on Day 28 (±7days) of each dosing cycle.

5. During treatment, if the medication is suspended due to toxicity within a treatment cycle, the subsequent safety assessment can be delayed accordingly. Tumor assessments will not be postponed accordingly and the assessment interval should be maintained at 4 to 8 weeks after the first dose date of each cycle as possible.

6. If the subject's 12-lead ECG results are abnormal, if the investigator considers it necessary, myocardial enzymes and echocardiography can be performed. In addition, the investigator may add unscheduled tests as clinically indicated.

7. Record adverse events, concomitant drugs and therapies.

8. In the second cycle and beyond, if a holiday is encountered, the corresponding dosing and safety visit schedule can be postponed until after the holiday.

B. Monotherapy extension period (period 2)

1. The investigational drug VG161 will be administered by multiple consecutive intratumoral injections, once daily, according to the recommended dose in Period 1 (including the number of consecutive administrations). Vital signs will be monitored daily at pre-dose (within-2h) and 2 h (± 15 min) and 4 h (± 15 min) post-dose.
2. Vital signs should be monitored at 24 h (± 1 h) and 48 h (± 1 h) after the last dose.
3. The following safety assessments will be performed weekly (D8, D15, D22) in Cycle 1, with a time window of ±1 day allowed.

1) Vital signs;
2) Brief physical examination;
3) Laboratory test: hematology, urinalysis and blood biochemistry;
4) 12-lead ECG.

4. The following assessments will be performed at the end of Cycle 1 (C1D28±1, but prior to dosing in Cycle 2):

1) Vital signs;
2) ECOG score;
3) Weight
4) Brief physical examination;
5) Laboratory test: hematology, urinalysis, blood biochemistry, coagulation function and thyroid function;
6) 12-lead ECG;

5. Starting from Cycle 2, vital signs will be monitored daily during dosing period and vital signs at pre-dose (within-2h) and 2 hours (±15 min) post-dose will be recorded. If the subject does not have vital sign abnormalities during the dosing period, vital sign test may be performed during the dosing period at the discretion of the investigator in subsequent cycles. The following safety visits will be performed at the end of each cycle (C1D28, C2 D28…); a time window of ±3 days is allowed:

1) Vital signs;
2) ECOG score;
3) Weight;
4) Brief physical examination;
5) Laboratory test: hematology, urinalysis and blood biochemistry;
6) Laboratory tests: coagulation function, thyroid function, at the end of every two cycles (C3D28, C5D28...);
7) 12-lead ECG.

6. Blood samples will be collected for pharmacodynamic and biomarker studies. The specific sampling time points and requirements are detailed in Section 7.2 (7.2 Pharmacodynamic and biomarker studies) and attached Table 2.
7. Tumor assessment: The tumor imaging examination will be performed initially at the end of the first cycle (C1D28±3days), and thereafter performed on Day 28 (±7days) of each dosing cycle.
8. During treatment, if the dose is interrupted due to toxicity, subsequent safety assessments may be delayed accordingly. Tumor assessments will not be postponed accordingly and the assessment interval should be maintained at 4 to 8 weeks as possible.
9. If the subject's 12-lead ECG results are abnormal, myocardial enzymes and echocardiography may be performed if deemed necessary by the investigator. In addition, the investigator may add unscheduled tests as clinically indicated.
10. Record adverse events, concomitant medications and treatments.

11. In the second cycle and beyond, in case of a holiday, the corresponding dosing and safety visit schedule can be postponed beyond the holiday.
12. If a subject achieves stable disease or partial response after 4 cycles, the SRC may decide whether to continue the treatment after discussion. Safety assessment visits and tumor assessments will be performed at the time points specified above.

8.2.3 End of Treatment visit (28 days after last dose)

The end of treatment visit (except death or lost to follow-up) must be completed within 28 days (± 7 days) after the last injection of the IP or before initiation of new anti-tumor therapy, whichever occurs first. Supposing the subject discontinues treatment since the toxicity does not resolve after postponing the dosing and the time of confirmed discontinuation of treatment is more than 28 days from the last injection of the IP, the evaluation items at the end of treatment visit should be completed within 7 days after discontinuing treatment. The examination items that have been performed within 7 days before the determination of discontinuation of treatment may not be repeated at the discretion of the investigator.

The specific assessment contents are as follows:

1) Vital signs;
2) Weight;
3) Comprehensive physical examination;
4) ECOG score;
5) Laboratory test: hematology, urinalysis, blood biochemistry, coagulation function and thyroid function;
6) Blood samples will be collected for pharmacodynamic and biomarker studies. The specific sampling time points and requirements are detailed in Section 7.2 (7.2 Pharmacodynamic and biomarker studies) and attached Table 2.
7) Pregnancy test for women of childbearing potential;
8) 12-lead ECG;
9) Tumor assessment (if the subject discontinues treatment for reasons other than tumor progression and has not undergone tumor assessment within 28 days, the tumor assessment should be performed at the time of treatment discontinuation);
10) Record concomitant drugs and therapies;
11) Record adverse events.

8.2.4 Safety follow-up

The occurrence of AE/SAE from the time the subject signed the informed consent form to 28 days after the last dose and AE/SAE whose causality to the IP could not be ruled out 28 to 90 days after the last dose should be recorded.

8.2.5 Survival follow-up

After completion of the end of treatment visit,PFS and OS follow-up is required.

For subjects who are out of the group not becourse of disease progress, PFS follow-up should be performed with imaging examination until disease progression or death or initiation of new anti-

tumor therapy (whichever occurred first); and imaging examination can be performed at study site or local hospital every 4 weeks (±7days).

OS follow-up can be performed by effective means such as telephone follow-up until death or loss follow-up, once at 3, 6, 9 and 12 months (±7days), until 1 year after the end of treatment visit.

9 Safety evaluations

Post-dose safety evaluation indicators include vital signs, ECOG score, physical examination, laboratory tests and adverse event evaluation. The frequency and severity of adverse events will be assessed according to the NCI CTCAE 5.0 criteria.

9.1 Adverse event

An adverse event (AE) is defined as any unintended symptom, sign, worsening disease, or other medical condition after administration of IPs, even if it is not related to the IP. "Investigational product" is defined as the product to be tested and the reference or placebo to be used in the trial.
A timely and complete AE report is helpful to the sponsor in determining whether an AE is associated with the drug in order to: ①learn more about drug toxicity; ②find out the dose-related toxicity of the drug; ③revise the protocol; ④ comply with laws and regulations to protect subjects, investigators and sponsors.
All adverse events should be documented and described on the adverse event page in the original medical records and case report form. The events may be observed from the time the subject signed the inform consent form to 28 days after the last dose, spontaneously reported by subjects, inquired by investigators, or identified by physical examination, laboratory tests, or other methods, or they may be related to the IP or not in the opinion of the investigator, and they may occur within 28 to 90 days after the last dose and whose causality with the IP could not be ruled out. If known, the name of the disease or disorder involved (i.e. diagnosis) should be recorded and the common terminology for adverse events refer to CTCAE 5.0. All adverse events should be observed until any of the following condition occurs: ①resolved; or ②stable; or ③return to baseline level; or ④ the event can be attributed to drugs other than the IP or factors unrelated to the behavior of the study, or when more information is unlikely to be available. If a subject withdraws from the trial and no further information about him/her is available, the time to withdraw should be recorded.
The investigator must also provide laboratory test results that are considered clinically significant or significantly abnormal to the sponsor and record them in the original medical documents and on AE pages of the case report form, regardless of whether they are related to the selected IP.
Medical conditions/diseases that have existed before the start of the study are considered an adverse event only if they worsen after the study.
The severity of an adverse event is a qualitative evaluation of the extent or severity of adverse events identified by the investigator or reported by the subject. The severity reflects neither the clinical seriousness of the event nor the causality with the IP, but describes the painfulness or extent and degree of occurrence only.
Severity grading and determination of adverse event was made based on the criteria of NCI CTCAE version 5.0.
The investigator should evaluate the possible relationship between the adverse event and the IP and the concomitant drug. According to the following criteria, the incidence of adverse reactions is calculated by counting the first 3 items as adverse reactions.

1. Related: Occurrence of AE and the use of IP have a plausible temporal relationship. AE is a known adverse reaction of IP. AE is alleviated or disappears after drug withdrawal and recurs after treatment is resumed. Subject's disease cannot explain the AE.
2. Probably related: Occurrence of AE and IP have a plausible temporal relationship. AE is a known adverse reaction of IP. AE is alleviated or disappears after treatment discontinuation, which subject's disease cannot explain. The result of re-challenge is unknown.
3. Possibly related: Occurrence of AE and the use of IP have a plausible temporal relationship. AE is a known or suspected adverse reaction of IP. However, other factors may cause the event, such as sickness, concomitant medications, etc. Result of the de-challenge is unknown, unclear or lack of decisive information.
4. Unlikely related: Occurrence of AE and the use of IP have a plausible temporal relationship, but the event is not a known adverse drug reaction and it is most likely to be caused by subject's disease or other treatments received.
5. Unrelated: Occurrence of AE and the use of IP do not have a plausible temporal relationship, e.g., the event has occurred before the use of IP. The AE is not a known adverse drug reaction, or the AE is determined to be caused by other factors, such as the subject's disease, other treatments or concomitant medications.

9.2 Serious adverse event

A serious adverse event (SAE) is defined as an adverse event that results in:

- death；
- life-threatening;
- hospitalization or prolongation of existing hospitalization;
- Results in persistent or significant disability or incapacity;
- Results in a congenital anomaly or birth defect;
- A medical event that the investigator believes to be a serious adverse event.

The following are NOT considered SAEs:

- Hospitalization for scheduled surgery or other treatment or for examination purpose before the study;

The investigator should immediately take appropriate treatment measures for the subjects to ensure their safety and must report to the sponsor in writing within 24 hours and then provide a detailed and written follow-up report in time in presence of an SAE that occurred from the time the subject signed the informed consent to 28 days after the last treatment, as well as an SAEs occurred 28 to 90 days after the last treatment whose causality with the IP could not be ruled out. It is crucial that the subject's identification code in the clinical study should be indicated in the serious adverse event report and follow-up report instead of the subject's personal identity information such asname, ID card number and address. The onset time, seriousness, duration, measures taken and outcome of the serious adverse events should be recorded on the serious adverse events record forms.

For death reports, other required information, such as autopsy reports and final medical reports, should be provided to the sponsor and the ethics committee by the investigator.

Table 11: Report Contact Information

Unit	Contact person	Tel.	Fax
CNBG-Virogin Biotech (Shanghai) Ltd.	Sun Changcheng	021-69152787	/
Clinical Trial Ethics Committee, the First Affiliated Hospital, Zhejiang University School of Medicine		0571-87236685	0571-87236685

Serious adverse events that are not resolved at the end of the trial or at early withdrawal of the subject must be followed up until any of the following condition is met:

- Event resolved
- Event stabilized
- Event returned to baseline level (if baseline value is available)
- The event can be attributed to drugs other than the IP or factors unrelated to the behavior of the study, or when more information is unlikely to be available (the patient or healthcare provider refuses to provide more information, or there is evidence that the patient is still lost to follow-up after every effort has been made).

9.3 Unexpected serious adverse reaction

An unexpected serious adverse reactions are all unintended and serious adverse reactions that are related to or suspected to be related to the IP, whose nature, severity, outcome and frequency is inconsistent with the expected risks as shown in current materials of the IP (e.g., Investigator's Brochure, etc.).

During the clinical trials of the drug, if the investigator has identified any unexpected and serious adverse reactions that are related or suspected to be related to the IP, and he/she should report them in an expedited way in the form of individual case safety reports following this standard and procedure following the Standards and Procedures for Expedited Reporting of Safety Data during Drug Clinical Trials. Individual case of unexpected serious adverse reaction reports should be following The ICH Guideline on Clinical Safety Data Management: Data Elements for Transmission of Individual Case Safety Reports E2B (R3). The terms should be coded using the ICH M1: Medical Dictionary for Regulatory Activities (MedDRA).

When there is any safety information of the clinical study from the sponsor, the investigator must sign upon receipt, read it carefully, and determine treatment for subjects on whether or not any changes should be made. It is important to talk to the patients as early as possible when necessary. Suspected unexpected serious adverse reactions offered by the sponsor must be reported to the ethics committee.

9.4 Pregnancy

If a female subject or the spouse of a male subject becomes pregnant during the treatment period and within 90 days after the last dose of IP, whichever occurs later, the investigator should

complete a pregnancy report form within 24 hours after becoming aware of the event, report to the sponsor in an expedited way and follow up on the outcome.

During the study, female subjects should discontinue the IP immediately if they became pregnant, and the investigator should be notified. The investigator should offer suggestions and discuss the risks of continuing pregnancy and possible influence on the fetus. The subject must withdraw from the study (premature discontinuation of IP). Continuous monitoring of the subjects should be performed until the end of pregnancy.

Any spontaneous or therapeutic abortion, birth defects or congenital malformations of the newborn, malformations and abnormalities of the stillbirth, complications of the mother and newborn, etc., if occurred during the pregnancy, should be reported as SAEs. The medical reason for the occurrence of the event during this process should be recorded as the AE terminology.

10 Efficacy evaluation

RECIST 1.1 is a standard method for measuring the response of solid tumours to treatment in clinical studies.

In 2008, the American Association for the Study of Liver Diseases (AASLD) proposed the concept of 'surviving tumour' in the design of clinical trials for hepatocellular carcinoma, and revised the RECIST criteria for evaluating the efficacy of immunotherapy, proposing the mRECIST criteria (Modified RECIST Criteria for Evaluation of Efficacy in Solid Tumours).

In 2010, the mRECIST evaluation guidelines were publicly published, and after 10 years of clinical trial validation, the mRECIST guidelines were updated in 2020 to further refine the evaluation criteria. The mRECIST criteria used in this trial were the evaluation criteria specified in the 2020 version of the guidelines (see Appendix 7 for details).

The new standard for judging the efficacy of tumor immunotherapy, iRECIST standard (Immune Response Evaluation Criteria In Solid Tumors), makes up for the shortcoming that RECIST or the modified WHO standard is not fully applicable to anti-tumor immunotherapy.

For IUPD on initial assessment by iRECIST criteria (first appearance of a new lesion or at least 20% greater than the nadir of the sum of the diameters of target lesions), the treatment and secondary evaluation may be continued in the absence of acute disease deterioration because shrinkage is likely to begin within 4 weeks after determination of iUPD. iUPD will be considered as iCPD only when there is an increase in tumor burden on two consecutive evaluations (4-8 weeks) and the absolute value of the sum of the measurements increases by at least 5 mm.

10.1 Efficacy endpoints

Objective response rate (ORR): Defined as the proportion of complete response (iCR) or partial response (iPR) (i.e., iCR+iPR) as the best time point response during the period from the start of the investigational drug dosing to the withdrawal from the trial;

Progression-free survival rate (PFS rate): defined as the proportion of patients in complete remission who do not relapse for a given period of time and the proportion of patients in partial remission or whose disease has not worsened who do not have disease progression for a given

period of time.

Progression-free survival (PFS): Defined as the time from the start of IP to the first occurrence of disease progression or death due to any cause.

Survival rate (OS rate): Defined as the proportion of subjects who are alive in the same test group from the start of the investigational drug dosing to the specified time point.

Overall survival (OS): Defined as the time from the start of the investigational drug dosing to the subject's death due to any cause.

Duration of response (DOR): Defined as the time from the first tumor assessment of iCR or iPR to the first assessment of iCPD or death due to any cause after the start of investigational drug dosing.

10.2 Efficacy evaluation

Baseline tumour assessment will be performed during the screening phase, and 1 tumour assessment will be performed at the end of each cycle. The efficacy endpoints of the dose-escalation phase of this trial, as well as the first phase of the Simon two-phase approach in the treatment extension phase, will be evaluated according to the iRECIST and RECIST 1.1 criteria, respectively, and also retrospectively in patients with HCC using the mRECIST criteria. The efficacy endpoints of the second phase of the Simon two-stage approach in the treatment extension phase will be evaluated according to the RECIST1.1 and mRECIST criteria, respectively, with reference to the iRECIST criteria. For each subject, specific lesions must be evaluated during the trial using the same evaluation techniques (e.g., enhanced CT, MRI). Evaluation sites should include the chest, abdomen, pelvis, and any other site suspected of having a tumour lesion. Considering the possibility of pseudoprogression with immunotherapy, if a subject first experiences disease progression (including the appearance of a new lesion), the investigator assesses that the subject has not experienced rapid tumour progression and the toxicity is tolerable, and the investigator believes that the subject would benefit from continued use of the test drug, the subject will be allowed to continue using the test drug and will be re-tested for tumour imaging in accordance with iRECIST at least 4 weeks (and no longer) after the first evaluation of disease progression. 8 weeks) for another tumour imaging evaluation until disease progression (by iRECIST criteria, iCPD) and the investigator determines that there is no benefit to be derived from continued treatment with the trial drug, or intolerable toxicity occurs, or voluntarily requests withdrawal, or otherwise terminates the study.

11 Statistical analysis

11.1 Analysis datasets

The analysis set for this trial will be based on the single agent dose escalation phase and the single agent treatment extension phase defined separately.

Single-agent dose-escalation phase

1) Safety Set (SS): all subjects who are enrolled, have used the trial drug at least once, and have a

post-dose safety record are part of the Safety Set. This data set is used for safety analysis.
2) Full Analysis Set (FAS): according to the principle of Intentional Analysis (ITT), the efficacy analysis is performed on all cases that are enrolled and have used the trial drug at least once.
3) Pharmacokinetic Analysis Set (PAS): all subjects enrolled, who have received at least one trial medication and have at least one PK data.
4) Dose-Limiting Toxicity (DLT) Evaluable Analysis Set: subjects who meet the minimum therapeutic requirements of the study and the safety evaluation requirements. That is, received VG161 administration as planned (≥ 80% of the planned dose) during Cycle 1 and completed safety follow-up throughout the DLT evaluation period or experienced a DLT.
5) Pharmacodynamic Analysis Set: all subjects enrolled, who have received at least one trial dose, and for whom at least one pharmacodynamic data is available.
Monotherapy Expansion Phase
1) Safety Set (SS): all subjects who are enrolled, have used the trial drug at least once, and have a post-dose safety record are part of the Safety Set. This data set is used for safety analysis.
2) Full Analysis Set (FAS): All cases enrolled and used at least once are analysed for efficacy according to the principle of Intentional Analysis (ITT).
3) Efficacy Evualable Analysis Set (EEAS): subjects with at least one post-baseline follow-up tumour assessment in the Full Analysis Set population.
4) Pharmacodynamic Analysis Set (PAS): all subjects enrolled, who have received at least one dose of the trial medication and have at least one dose of pharmacodynamic data.

11.2 Statistical methods

The statistical analysis plan (SAP) should be prepared after the protocol finalization and completed before database lock. The SAP will specifically define and describe all planned statistical analyses per the protocol's characteristics.
This study is based on descriptive statistical analyses, which will be summarised based on different phases and different dose groups if not specified. The statistical items mainly include baseline indicators, safety evaluation, pharmacokinetics/viral excretion/serum antibody indicators and efficacy indicators. The main statistical indicators for quantitative data include arithmetic mean, standard deviation, median, minimum and maximum values; and for categorical data, frequency and percentage.
1) Subject baseline parameters
Baseline parameters include demographic characteristics and previous treatment history and will be analyzed using descriptive statistics.
2) Safety parameters
The parameters include adverse events, vital signs, ECOG score, 12-lead ECG and laboratory tests. Adverse events will be classified and graded according to CTCAE v5.0 and the number of occurrences of adverse events and the number/percentage of subjects will be summarized separately. Drug-related adverse events, Grade 3 or higher adverse events, immune-related adverse

events (irAEs), serious adverse events will be summarized separately. Vital signs, ECOG score, 12-lead ECG and laboratory values at each time point will be summarized using descriptive statistics.

3) Pharmacokinetics/virus shedding/serum antibody

The parameters include the distribution and excretion of HSV-1 after local administration and the blood concentration of PD-L1 blocking polypeptide and IL12 and serum antibodies. PK parameters will be analyzed descriptively.

4) Efficacy parameters

Point estimates of ORR were calculated and 90% (one-sided 95%) confidence intervals were calculated based on the Clopper-Pearson method. OS and PFS, DOR were estimated for all subjects and dose groups by Kaplan-Meier method, median time and its 95% confidence interval were estimated and plotted. PFS/OS rates were calculated and their 95% CIs were estimated by the Greenwood formula.

5) Pharmacodynamic parameters and biomarker parameters

Preliminary evaluation of changes in immune-related indicators such as blood lymphocyte subsets, cytokines and T cell before and after administration, and preliminary analysis of the correlation with efficacy.

12 Data management and quality control

12.1 Data management

The electronic data management system will be adopted in this trial, and the electronic case report form (eCRF) will also be constructed for directly input of clinical trial data. The investigator should promptly and accurately enter data from the study medical records into the eCRFs. The CRA should monitor the condition through eCRF, and can ask questions online at any time when any problem is found, and receive the answer from the investigator online to modify incorrect data, and can repeatedly ask questions if necessary. The CRA should review and confirm each data in the eCRF. After each subject has completed the trial and passed the review conducted by the CRA, the data manager will lock the data. After all the data are locked, the data manager will export the data and transfer it to the statistician for statistical analysis. To ensure data security, the persons responsible for data input, monitoring and analysis should be granted with authorized accounts and endowed with different authorities according to their roles. Irrelevant personnel is prohibited to access and modify data.

CRF/eCRF will not be used as the original record of the trial. The investigator should keep the subjects' medical records, clinical records, and laboratory test records as the original source documents of the study to ensure the traceability of the data.

12.2 Monitoring

The CRA should be delegated by the sponsor and should contact the site before the start of the study to confirm that the site has established the appropriate conditions in terms of staffing and

laboratory equipment, etc., and participate in the review of the protocol, study requirements, standard operating procedures, and the sponsor's requirements together with the site personnel.
The CRA shall regularly monitor the work of the site, and conduct regular monitoring when enrolling the first subject, during the study and at different stages after the end of the study.
The monitor should confirm whether the clinical trial is conducted in strict accordance with the clinical study protocol, the authenticity and accuracy of clinical materials and data, whether the safety and rights of subjects are protected, and the requirements stipulated by other laws and regulations; and solve problems that arise during the trial together with the investigator if necessary.

12.3 Preservation of data

The investigator should adequately keep the original data obtained from the trial. According to the principles of Chinese GCP, all original data of the clinical trial should be kept by investigators until 5 years after the termination of the clinical trial.

13 Ethical requirements

13.1 Ethics Committees

The investigator's brochure, protocol, informed consent form, case report form, approval of drug clinical trial (if any), the certificate of analysis of IPt and any other information for subjects (ads for recruiting subjects, subject's compensation protocol, etc.) must be submitted to the ethics committee for review and approval before the start of the trial. The approval by the Ethics Committee should be attached with a list of all the Committee members involved in the approval documents and their respective duties.
During the clinical study, any issues related to the safety of the clinical study, such as modification to the clinical study protocol or the informed consent form and serious adverse events in the clinical study, must be reported to the Ethics Committee promptly. The completion or early termination of the clinical study must also be reported to the EC.

13.2 Informed consent

The investigator must explain the trail nature, purpose, procedures, schedule, potential risks and benefits, and any discomfort to each subject in the informed consent. Each subject must know that participation in the trial is voluntary and that he/she can withdraw from the trial at any time as well as withdraw consent without prejudice to his/her subsequent treatment or relationship with the treating physician.
The informed consent form should be given in a standard written format and in non-professional languages where possible, so that subjects may fully understand the potential risks and benefits of participating in the trial. The subject/legal representative should have sufficient time to consider and ask questions,
Before participating in the study, all subjects will be asked to sign and date the informed consent form after the investigator has explained the essential contents of the study to them and has confirmed that they understand the purpose of the study. If the subject is unable to sign, he/she

may provide a verbal statement of consent in the presence of a witness (a third party unrelated to the sponsor and the investigator), and the investigator and witness who are responsible for the informed consent should sign for confirmation. The signed informed consent form must be kept in the documents at the site, and a copy of the signed informed consent form will be provided for the subject/legal representative. The subject cannot enter the trial before obtaining the informed consent and signing the informed consent form.

13.3 Subject's privacy

The investigator must ensure that subjects' privacy is protected in clinical trials. In all documents submitted to the sponsor, the subject's identity enrolled in the clinical trial can only be recognized by the trial patient number and initials rather than full names of subjects. The investigator must adequately keep an enrollment log showing subject names and addresses. This enrollment log must be kept strictly confidential by the investigator and should not be disclosed to the sponsor.

14 Responsibilities & regulations

1) Sponsor

The sponsor shall provide the site with a trial-related investigator's manual and other materials and trial funds. The sponsor shall also provide free investigation drugs that have passed the tests, copies of trial-related materials (including but not limited to the drug clinical trial approval notice [or other regulatory authority approval documents], the sponsor's business license, production qualification certificates), and the original certificate of analysis. The sponsor shall establish a quality assurance system for clinical trials, appoint the CRA that the investigator accepts, and organize audits of clinical trials when necessary. The sponsor shall promptly analyze the safety information reported by the investigator and take necessary measures to ensure the rights and interests of subjects.

2) Investigator

All information provided by the sponsor shall be kept strictly confidential by the study site, while other test participants and the Ethics Committee shall be required to take the same confidentiality measures. The information provided to the site shall not be disclosed to others without the written permission of the sponsor. The investigator must read and understand the protocol's contents in detail and perform the study strictly following the protocol. The investigator is responsible for making medical decisions related to the clinical trial, taking necessary measures to ensure the safety of subjects, and recording the clinical trial data in the medical records and CRFs in a timely, accurate and complete manner. The investigator should receive the inspections or audits conducted by CRA or auditors sent by the sponsor and inspections conducted by drug regulatory authorities to ensure the quality of clinical trials.

15 Publications

All data and test results are the properties of CNBG-Virogin Biotech (Shanghai) Ltd. The investigator will not publish them without the consent of the sponsor.

The intellectual property rights of confidential information only belong to CNBG-Virogin Biotech (Shanghai) Ltd. and shall not be disclosed to others without the written consent of CNBG-Virogin Biotech (Shanghai) Ltd. or used for intentions except for this study.

16 References

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17 Appendices

Appendix 1. Study Schedule

Date / (Time window) / Items	Screening/baseline		Cycle 1(28 days)						Cycle 2 (28 days)		Cycle 3 and thereafter (28 days)		End of treatment visit (28 days post-dose)	Survival follow-up[h] (each 3months)
	-		-	± 1 day					± 7 day	± 3 days	± 7 days		± 7 days	± 7 days
	-28 to -8	-7 to -1	D1	D2-3*	D8	D15	D22		D1-D3*	D28	D1-D3*	D28		
Signed informed consent[a]	X													
Review of inclusion and exclusion criteria	X													
Demographic data	X													
Medical history and medication history	X													
Vital signs[b]		X	X	X	X	X	X	X	X	X	Remarks[b]	X	X	
Body height		X												
Weight		X						X		X		X	X	
Complete physical examination		X											X	
Brief physical examination					X	X	X	X		X		X		
ECOG score		X						X		X		X	X	
Hematology		X			X	X	X	X		X		X	X	
Urinalysis		X			X	X	X	X		X		X	X	
Coagulation function[c]		X						X				X[c]	X	
Blood Chemistry		X			X	X	X	X		X		X	X	
Thyroid function[c]		X						X				X[c]	X	
Pregnancy test[d]		X											X	

Infectious disease markers	X												
Herpes simplex type I antibody	X												
12-lead ECG	X			X	X	X	X		X		X	X	
Echocardiogram[e]	X	as clinically indicated											
Cardiac enzyme[e]		as clinically indicated											
Tumor assessments[i]	X						X		X[i]		X[i]	X	X
Study medication administration		X	X					X		X			
PK/virus excretion[f]		See Appendix Table 2 for details (Period 1 Cycle 1 only)											
Biomarker blood sampling		See Appendix table 2 for details											
Concomitant drugs and therapies	X	X	X	X	X	X	X	X	X	X	X	X	
Adverse events[g]	X	X	X	X	X	X	X	X	X	X	X	X	

*: Days 2-3 dosing and follow-up schedules in Period 1. Single-agent Dose-escalation Period vary depending on the dose group, and in Period 2. Single-agent Extension Period, a fixed number of consecutive dosing days will be determined according to the recommended number of consecutive doses in Period 1. Refer to Section 8.2.

a. An informed consent form should be signed by each patient before the initiation of any study-related procedure.

b. Period 1. Single-agent Dose-escalation Period:

For a single day dosing group: monitor vital signs before (-2h) and 2h (±15min), 4h (±15min), 24h (±1h), 48h (±1h) after C1D1 intratumoral injection;

For a multi-day dosing group: monitor vital signs before (within-2h) and 2h (± 15 min), 4h (± 15 min) after dosing, and 24h (± 1 h), 48h (± 1 h) after the last dose.

Vital signs are monitored and recorded daily at before dosing (within -2h) and 2 hours (±15 min) after dosing in Cycle 2 of injection. If a subject has not shown abnormality in vital signs during the dosing period, his/her vital signs may be tested at the discretion of the investigator in subsequent cycles.

Period 2. Monotherapy Extension Period:

Vital signs will be monitored at pre-dose (within -2h) on the first day and 2 h (± 15 min) and 4 h (± 15 min) post-dose, and at 24 h (± 1 h) and 48 h (± 1 h) after the last dose.

Starting from Cycle 2, vital signs will be monitored daily during dosing period and vital signs at pre-dose (within -2h) and 2 hours (±15 min) post-dose will be recorded. If a subject has not shown abnormality in vital signs during the dosing period, his/her vital signs may be tested at the discretion of the investigator in subsequent cycles.

c. Coagulation and thyroid function tests will be performed at screening, C1D28, C3D28, and end of treatment visit.

d. A blood pregnancy test is required only for women of childbearing potential.

e. The echocardiography is required at screening; during the treatment period, if the subject has an abnormal 12-lead ECG, myocardial enzyme test and echocardiography

may be conducted as deemed necessary by the investigator.

f. The PK/viral excretion test will be performed only at Cycle 1 of Period 1. Single-agent Dose-escalation Period.

g. The information about all adverse events from the time of signing the informed consent form to 28 days after the last dose, and adverse events whose causality to the IP could not be ruled out 28 to 90 days after the last dose should be followed up and recorded.

h. After completion of the end of treatment visit,PFS and OS follow-up is required. For subjects who are out of the group not becourse of disease progress, PFS follow-up should be performed with imaging examination until disease progression or death or initiation of new anti-tumor therapy (whichever occurred first); and imaging examination can be performed at study site or local hospital every 4 weeks (±7days). OS follow-up can be performed by effective means such as telephone follow-up until death or loss follow-up, once at 3, 6, 9 and 12 months (±7days), until 1 year after the end of treatment visit.

i. Tumor assessment: Tumor imaging examination will be performed at the end of the first cycle (C1D28), and thereafter performed on Day 28 (±7days) of each dosing cycle

Appendix 2: Pharmacokinetics/Virus Excretion/Biomarker Sample Collection

Phase 1. Single Dose-escalation Phase

2.1 One-day dosing group: Period 1.

Trials days	Blood samples collection points (except serum antibodies) (time window)	Blood samples collection points (serum antibody) (time window)	Urine sample collection points (time window)	Fecal sample collection points (time window)	Saliva (oral) swab collection points (time window)	Superficial lesion injection site wiping sampling (time window)	Pharmacodynamic and biomarker blood sampling points (time window)
C1D-2		Pre-dose (-2 days)		Pre-dose (-2 days)			Pre-dose (-2 days)
C1D1	Pre-dose (within-2h)		Pre-dose (within -2h)		Pre-dose (-within 2h)	Pre-dose (within -2h)	
				Retain sample for each feces up to 48 h post-dose	0.5h (±5min)	0.5h (±10min)	
	1h (±5min)				1h (±5min)	1h (±10min)	
	2h (±10min)						
	6h (±10min)				4h (±10min)	4h (±10min)	
					8h (±10min)	8h (±10min)	
	12h (±10min)	12h (±10min)					
C1D2	24h (±1h)	24h (±1h)	On the morning of the day		24h (±1h)	24h (±1h)	24h (±1h)
C1D3	48h (±2h)	48h (±2h)					
C1D5	96h (±2h)	96h (±2h)					
C1D8	168h (±2h)	168h (±2h)					
C1D15	On the morning of the day (± 1 day)	On the morning of the day (± 1 day)	On the morning of the day (± 1 day)		On the morning of the day (± 1 day)		On the morning of the day (± 1 day)
C1D22	On the morning of the day (± 1 day)	On the morning of the day (± 1 day)	On the morning of the day (± 1 day)				
C1D28	On the morning of the day (±1 day, pre-dose on C2D1)	On the morning of the day (±1 day, pre-dose on C2D1)	On the morning of the day (±1 day, pre-dose on C2D1)		On the morning of the day (±1 day, pre-dose on C2D1)		On the morning of the day (±1 day, pre-dose on C2D1)

2.2 Two-day dosing group: Period 1.

Trials days	Blood samples collection points (except serum antibodies) (time window)	Blood samples collection points (serum antibody) (time window)	Urine sample collection points (time window)	Fecal sample collection points (time window)	Saliva (oral) swab collection points (time window)	Superficial lesion injection site wiping sampling (time window)	Pharmacodynamic and biomarker blood sampling points (time window)
C1D1	Pre-dose (within -2h)	Pre-dose (within -2h)	Pre-dose (within -2h)	Pre-dose (-2 days)	Pre-dose (within -2h2h)	Pre-dose (within -2h)	Pre-dose (within -2h)
C1D2*	Pre-dose (within -2h)		Pre-dose (-2h)		Pre-dose (within -2h)	Pre-dose (within -2h)	Pre-dose (within -2h)
				Retain sample for each feces up to 48 h post-dose on D2	0.5h (±5min)	0.5h (±10min)	
	1h (±5min)				1h (±5min)	1h (±10min)	
	2h (±10min)						
	6h (±10min)				4h (±10min)	4h (±10min)	
					8h (±10min)	8h (±10min)	
	12h (±10min)	12h (±10min)					
C1D3	24h (±1h)	24h (±1h)	On the morning of the day		24h (±1h)	24h (±1h)	24h (±1h)
C1D4	48h (±2h)	48h (±2h)					
C1D6	96h (±2h)	96h (±2h)					
C1D9	168h (±2h)	168h (±2h)					
C1D15	On the morning of the day (± 1 day)	On the morning of the day (± 1 day)	On the morning of the day (± 1 day)		On the morning of the day (± 1 day)		On the morning of the day (± 1 day)
C1D22	On the morning of the day (± 1 day)	On the morning of the day (± 1 day)	On the morning of the day (± 1 day)				
C1D28	On the morning of the day (±1 day, pre-dose on C2D1)	On the morning of the day (±1 day, pre-dose on C2D1)	On the morning of the day (±1 day, pre-dose on C2D1)		On the morning of the day (±1 day, pre-dose on C2D1)		On the morning of the day (±1 day, pre-dose on C2D1)

Note: * If the second dose is administered every other day, it is actually the third day of the course.

2.3 Three-day dosing group: Period 1.

Test days	Blood samples collection points (except serum antibodies) (time window)	Blood samples collection points (serum antibody) (time window)	Urine sample collection points (time window)	Fecal sample collection points (time window)	Saliva (oral) swab collection points (time window)	Superficial lesion injection site wiping sampling (time window)	Pharmacodynamic and biomarker blood sampling points (time window)
C1D1	Pre-dose (within2h)	Pre-dose (within-2h)	Pre-dose (within -2h)	Pre-dose (-2 days)	Pre-dose (within -2h)	Pre-dose (within -2h)	Pre-dose (within -2h)
C1D2*					Pre-dose (within -2h)		
C1D3*	Pre-dose (within -2h)		Pre-dose (within -2h)		Pre-dose (within -2h)	Pre-dose (within -2h)	Pre-dose (within -2h)
				Retain sample for each feces up to 48 h post-dose on D3	0.5h (±5min)	0.5h (±10min)	
	1h (±5min)				1h (±5min)	1h (±10min)	
	2h (±10min)						
	6h (±10min)				4h (±10min)	4h (±10min)	
					8h (±10min)	8h (±10min)	
	12h (±10min)	12h (±10min)					
C1D4	24h (±1h)	24h (±1h)	On the morning of the day		24h (±1h)	24h (±1h)	24h (±1h)
C1D5	48h (±2h)	48h (±2h)					
C1D7	96h (±2h)	96h (±2h)					
C1D10	168h (±2h)	168h (±2h)					
C1D15	On the morning of the day (± 1 day)	On the morning of the day (± 1 day)	On the morning of the day (± 1 day)		On the morning of the day (± 1 day)		On the morning of the day (± 1 day)
C1D22	On the morning of the day (± 1 day)	On the morning of the day (± 1 day)	On the morning of the day (± 1 day)				
C1D28	On the morning of the day (±1 day, pre-dose on C2D1)	On the morning of the day (±1 day, pre-dose on C2D1)	On the morning of the day (±1 day, pre-dose on C2D1)		On the morning of the day (±1 day, pre-dose on C2D1)		On the morning of the day (±1 day, pre-dose on C2D1)

Note: *If the drug is administered every other day between the second and/or the third, it is actually Day 3 and Day 4/5 of the course of treatment.

Cycles in Period 2. Monotherapy Extension Period and cycle 2 and subsequent cycles in Period 1. Single-agent Dose-escalation Period (dosing for 3 consecutive days scheduled tentatively)

2.6 Tentative 3-day dosing group: Cycles in Period 2 and cycle 2 and subsequent cycles in Period 1

Test days (per cycle)	Pharmacodynamic and biomarker blood sampling points (Time window)
D1	Pre-dose (within-2h) on Day 1 of each cycle
D6	24h (±1h)
D15	On the morning of the day (± 1 day)
D28	On the morning of the day (± 1 day, prior to dosing of next cycle)

Appendix 3: ECOG performance status score

Grade	Performance Status
0	Fully active, able to carry on all pre-disease performance without restriction.
1	Restricted in physically strenuous activity but ambulatory and able to carry out work of a light or sedentary nature, e.g., light house work, office work.
2	Ambulatory and capable of all self-care but unable to carry out any work activities. Up and about more than 50%of waking hours
3	Capable of only limited self-care, confined to bed or chair more than 50% of waking hours.
4	Confined to bed and unable to carry on any self-care.

Appendix 4: New York Heart Association (NYHA) Functional Classification

Grade	Patient Symptoms
Grade I	The patient has organic heart disease, but no limitation of physical activity. Ordinary activity does not cause fatigue, palpitation, dyspnea or angina pectoris.
Grade II	Patients have slight limitation of physical activity. Comfortable at rest. Ordinary activity results in fatigue, palpitation, dyspnea or angina pectoris.
Grade III	Patients have marked limitation of physical activity, and less than ordinary activity causes fatigue, palpitation, dyspnea or anginal pain.
Grade IV	Patients are unable to carry out any physical activity without discomfort. Symptoms of cardiac insufficiency at rest. If any physical activity is undertaken, discomfort is increased.

Appendix 5: Excerpts from the interpretation of Immune Response Evaluation Criteria in Solid Tumors (iRECIST)

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Research Process of Unconventional Response of Solid Tumor Immunotherapy and Its Response Evaluation Criteria

Development of unconventional response and related efficacy evaluation of ICIs in the treatment of solid tumor

Liu Ya's review: reviewed by Deng Hongbin (Department of Oncology, the First Affiliated Hospital of Chongqing Medical University, Chongqing 400000)

[Abstract] With the development of immunology and molecular biology, immunotherapy has become a new model of anti-tumor therapy. In particular, the clinical application of immune checkpoint inhibitors has become increasingly widespread, and it has shown excellent prospects in the treatment of a variety of solid tumors, and is regarded as one of the most potential anti-tumor treatments. However, more and more evidence suggest that immunotherapy has produced unconventional immune response patterns. These patterns are different from traditional response patterns, including pseudoprogression and delayed response. The studies have also confirmed that traditional response evaluation criteria in solid tumors usually cannot accurately capture such unconventional response, making it difficult to correctly evaluate the efficacy of immunotherapy, which has brought great difficulties and challenges to clinical work. Therefore, new immunotherapy efficacy evaluation criteria in solid tumors need to be established and improved urgently, leading to a series of immune-related response evaluation criteria being proposed one after another. This article reviews the research development and application of unconventional response patterns of solid tumor immunotherapy, mainly checkpoint inhibitor therapy and related response evaluation criteria.

[Keywords] Immunotherapy; solid tumors; pseudoprogression; hyperprogression; delayed response; response evaluation criteria

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Immunotherapy, especially immune-checkpoint inhibitors (ICIs) therapy, significantly improves the therapeutic efficacy of advanced tumors by relieving immunosuppression, improving the immune microenvironment, and enhancing effective immune infiltration. This "paradigm shift" change in treatment is accompanied by the emergence of unconventional response patterns, pseudoprogression, delayed response or hyperprogression disease (HPD), etc. [1] Recently, a number of clinical studies have suggested, [2] the traditional response evaluation criteria in solid

tumors based on RECIST v1.1 underestimate the clinical efficacy of immunotherapy. Among them, the evaluation of progression-free survival (PFS) and objective response rate (ORR) is also inaccurate, which often leads to early interruption of treatment, affecting survival benefits. This brings considerable changes to the selection of clinical treatment decisions and the setting of related clinical trial observation indicators and outcomes. Therefore, a series of immunotherapy efficacy evaluation criteria in solid tumors have been proposed one after another and have been continuously revised and improved in clinical practice. This article focuses on ICIs treatment and reviews the current research and development and application status of unconventional response patterns of solid tumor immunotherapy and related response evaluation criteria, hoping to provide a specific reference for clinical practice.

1 Unconventional response in immunotherapy of solid tumors

1.1 Basic concepts

Traditional cytotoxic drugs and targeted drugs directly kill tumor cells to shrink the lesion. Immunotherapy suppresses tumor cells by activating immune cells, enhancing immune infiltration, and maintaining effective local immune responses. In immunotherapy, some patients do not achieve tumor shrinkage immediately. On the contrary, the lesions are enlarged transiently at the beginning of the treatment, or even new lesions appear, and then the lesions shrink or disappear; or after a period of treatment, the lesions persist stably, but the patient's survival is prolonged; also, there are also phenomena of rapid deterioration of lesions and significant shortening of OS within a short period of immunotherapy, which are different from the traditional response pattern, that is, the unconventional response patterns. [1] Among them, pseudoprogression and delayed response can be regarded as effective objective responses in the course of immunotherapy, while hyperprogression will lead to a rapid deterioration of the disease and threaten the life of the patient. Therefore, accurately identifying hyperprogression and correctly evaluating the efficacy of immunotherapy is of great significance to clinical work. [3]

1.2 Common types

(1) Pseudoprogression

Pseudoprogression means that new lesions occur or the original lesions are enlarged in the early stage of immunotherapy, and the lesions stabilize or shrink in the later stage. The molecular basis is that in the early stage of medication, T cell proliferation and immune activation cause local enlargement of the lesion and even new infiltration foci. [4] This phenomenon is confirmed by local puncture and biopsy after ipilimumab monotherapy for advanced melanoma progression, suggesting local immune cell infiltration and necrosis. [5] The currently reported pseudoprogression incidence is less than 10%, and the most common pseudoprogression is seen

in melanoma and NSCLC. [3]

(2) Delayed response

A delayed response is a phenomenon that due to the infiltration of immune cells and the continuous occurrence of specific immune responses, the lesions continue to stabilize or shrink after a period of immunotherapy or even after stopping treatment and bring survival benefits. [4,6] This phenomenon occurs in 10% to 30% of patients treated with single-agent ICIs, and is often seen in melanoma and renal cell carcinoma. Accurate assessment of delayed response is undoubtedly conducive to setting the duration of immunotherapy. [2,6]

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(3) Hyperprogression disease

HPD refers to the rapid increase of lesions in a short period of time after immunotherapy. It is another typical unconventional response pattern that occurs during tumor immunotherapy and violates the normal growth law of tumors. Due to the varying evaluation methods of HPD, the incidence of HPD reported clinically fluctuates between 9%-29%. [7] Once HPD occurs, the median OS of patients is about 3 months. [8,9] The cause of its occurrence is not yet clear, mainly including molecular biological factors and clinical factors. Such as overexpression of CD4 + Treg cells, compensatory T cell depletion, FOXP3 + Treg cells and MDM2 expansion, EGFR mutations, IDO, IFNγ and certain cytokines, TTF (time to treatment failure) < 2 months, autoimmune function before immunotherapy, etc. However, the number of lesions before treatment, low-performance status (PS), disease stage, lymphocyte count are not considered causes of HPD. [8,10]

1.3 Traditional response evaluation criteria in solid tumors need to be revised urgently

Given the unconventional responses of immunotherapy, relevant clinical experience [11] indicates that the time to response of immunotherapy is later than that of traditional chemotherapy. The response may even appear after evaluation of "progression" based on conventional chemotherapy criteria (WHO, RECIST criteria, etc.); before it is possible to judge whether it is a "true progression", the presence of "progression" without clinical symptoms is allowed, and it is inappropriate to interrupt immunotherapy rashly. However, hyperprogression is accompanied by a sharp decline in OS, and timely identification and change of treatment are necessary. [3] Traditional efficacy evaluation criteria have suggested that they cannot accurately capture and identify, and often underestimate the efficacy of immunotherapy. This has prompted the continuous production, revision and improvement of various immune-related response evaluation criteria.

2 Establishment and Development of Immune Response Evaluation Criteria in Solid Tumors

2.1 Evaluation criteria for pseudoprogression and delayed response

(1) Immune-related response criteria (irRC)

Before the emergence of immunotherapy, chemotherapy with cytotoxic drugs was the main way to treat advanced solid tumors. Its response evaluation criteria were updated from the dual-diameter measurement of the WHO criteria [12] and the single-diameter measurement of the RECIST1.0 criteria [13] to RECIST v1.1 criteria, [14] the effectiveness of treatment is manifested as the shrinkage of the primary tumor or no new lesions. [15] Therefore, once the primary tumor has been enlarged significantly or new lesions occur, it will be directly rated as progression disease (PD), and the clinical medication regimen will be therefore adjusted. In the clinical practice of

using the criteria to evaluate immunotherapy for solid tumors, it is found that none of the previous evaluation criteria can fully reflect the true clinical response to immunotherapy. In 2009, WOLCHOK et al. [16] proposed the irRC criteria based on the WHO criteria, and proved the feasibility of the evaluation criteria through a clinical trial of ipilimumab in the treatment of advanced melanoma in 487 patients. The incidence of pseudoprogression reported in the trial was about 10%. It was subsequently reported [17-19] that the incidence of pseudo-progression in other solid tumors was 1%-5%. The difference between the irRC criteria and the WHO criteria is that the tumor burden (TB) is introduced for the first time, and new measurable lesions (≥5mm×5mm) are included in the total tumor burden (TTB) and compared with the baseline. The initial observation of new lesions is not immediately considered progression disease and are divided into new measurable lesions and new non-measurable lesions; measurable new lesions are included in TTB, while non-measurable lesions are not meaningful for evaluation; assessment will be performed again at least 4 weeks later, and 2 consecutive TTB increases of more than 25% will be defined as PD. As the first immune-related response evaluation criteria, the irRC criteria have achieved certain results in clinical application. A clinical trial based on phase Ib KEYNOTE-001 [20] used RECIST and irRC criteria to evaluate the clinical efficacy of pembrolizumab. The results showed that the RECIST criteria underestimated the clinical benefit of approximately 15% of patients (because of pseudoprogression and delayed response). Compared with the traditional evaluation criteria, the irRC criteria is more accurate, but requires a large number of lesions to be measured, and the dual-diameter measurement is complicated and its repeatability is not high, especially when the changes in the lesions are small, it is easy to exaggerate the actual changes of the lesions to lead to an assessment of PD. [21-22]

(2) Immune-related response evaluation criteria in solid tumors (irRECIST)

In 2013, NISHINO et al. [23] randomly selected 25 patients with advanced melanoma who received ipilimumab treatment, used single-diameter method and dual-diameter method to measure the lesions respectively, and use the irRC criteria as the lesion selection and efficacy evaluation criteria, confirming one-dimensional irRC criteria and the two-dimensional irRC criteria were highly consistent in the evaluation of treatment response. Based on this, at the 2014 European Society for Medical Oncology (ESMO) conference, the irRECIST criteria based on single diameter measurement were proposed. [24] This criteria select target lesions and define PD with RECIST v1.1 criteria as a reference, and define the sum of long diameters of target lesions as TB and TTB, pointing out that non-target lesions and new lesions have reference value, and the initial assessment of PD still needs to be confirmed by reassessment at least 4 weeks later. However, the irRECIST criteria do not give clear requirements on how to reflect the specific value of non-target

lesions and new lesions. [25] In fact, the irRECIST criteria have no significant breakthrough compared to the irRC criteria, and it is rarely used in clinical practice. Only the analysis of the unconventional response phenomenon in a phase II clinical trial of nivolumab in the treatment of metastatic clear cell renal cell carcinoma (MccRCC) [26] showed that the response assessment taking into account biomarkers demonstrated that the irRECIST criteria was better than the traditional RECIST criteria with regards to capturing the unconventional clinical response of immunotherapy. Although the irRC criteria reduces the determination of pseudoprogression and delayed response to a certain extent, it is derived from and mainly used in the response evaluation in melanoma, and existing data show that the incidence of unconventional responses varies in various solid tumors. So, is the criteria generally applicable to other solid tumors that lack large sample size randomized studies? How to define the specific value of new lesions in response evaluation? When is it more appropriate to initially evaluate the response to ICIs in the treatment of various solid tumors? It is the existence of these problems that promote the continuous revision of immunotherapy evaluation criteria.

(3) Immune response evaluation criteria in solid tumors (iRECIST)

In 2017, the RECIST working group proposed new immunotherapy efficacy evaluation criteria, namely iRECIST criteria. The original intention of the criteria is to provide a consistent reference standard for clinical trials related to immunotherapy. [25] The iRECIST criteria have some modifications to the RECIST v1.1 criteria, and the evaluation indicators include immune complete response (iC'R), immune partial response (iPR), and immune stable disease (iSD), immune unconfirmed progression disease (iUPD), and immune confirmed progression disease (iCPD). The main difference from the RECIST v1.1 criteria is that at the initial evaluation, the PD evaluated by the RECIST v1.1 criteria is defined as iUPD. For new lesions, target lesions are selected according to the RECIST v1.1 criteria and divided into new target lesions and new non-target lesions. Unlike the irRC criteria, new target lesions are not included in TTB. Instead, they are used separately as indicators for subsequent evaluation. The initial evaluation of iUPD needs to be re-confirmed after 4-8 weeks. At the time of reevaluation, enlargement of the target lesion and the new target lesion by ≥5 mm, any progression of the new non-target lesion or the occurrence of any new lesion will be considered as iCPDt; conversely, if the lesions do not change significantly and do not meet the iCR, iPR, iSD per RECIST v1.1 criteria, then the evaluation continues to be iUPD. If the lesion achieves iCR, iPR, and iSD evaluated by RECISTv1.1, the baseline needs to be re-adjusted to the reduced tumor burden, and the time from the start of treatment to the occurrence of any of the above definitive responses is defined as time to the best response to immunotherapy (immune best overall response, IBOR). The criteria specifically point out that if the RECIST v1.1 criteria

evaluate as PD and the iRECIST criteria evaluate as iUPD, the patient's clinical symptoms should be considered. If the clinical manifestations are stable, consider continuing to use the current immunotherapy, but the re-examination time should be appropriately shortened to 4-8 weeks. Even if iRECIST evaluates as iCPD, immunotherapy can be continued if the patient and family members are willing. For those patients who discontinue treatment due to iCPD, regular follow-up is also necessary, which will help observers further understand the law of tumor growth and development with immunotherapy. The iRECIST criteria defines iBOR for the first time. The reconfirmation evaluation method for unconventional response patterns such as pseudoprogression and delayed response in the treatment of solid tumors with ICIs is scientific. Besides, the value of new lesions and non-target lesions is clarified. It was emphasized that the response evaluation of ICIs treatment is not only determined by imaging findings, also, it is essential to take account into clinical symptoms.

TAZDAH et al. [17] compared the responses based on RECIST1.1 criteria, irRECIST criteria, and iRECIST criteria in a retrospective study that included 160 patients with advanced NSCLC treated with PD-I/PD-LI, and found that unconventional responses accounted for 13%, RECIST1.1 criteria underestimated 11% of patients' clinical benefit. The irRECIST criteria and iRECIST criteria were highly consistent.

However, another prospective clinical trial that included 42 patients with advanced NSCLC treated with PD-1/PD-L1 demonstrated that [27] the inconsistency rate between the iRECIST criteria and the RECISTv1.1 criteria evaluations was only 2/42, and the difference was not statistically significant; the iRECIST criteria was consistent with the RECIST v1.1 criteria. Therefore, the immune-related response criteria still need to be verified by randomized clinical studies. In addition, the dual-node uncertainty evaluation method of the iRECIST criteria focuses on the investigation of pseudoprogression and delayed response and may ignore the hyperprogression disease.

(4) Immune-modified response evaluation criteria in solid tumors (imRECIST)

In 2018, to implement atezolizumab-related clinical trials, HODI et al. [1] innovatively proposed the imRECIST criteria, which are the RECISTv1.1 criteria with modifications to the irRC criteria. The difference from the previous immune-related response evaluation criteria is that the criteria are proposed mainly for imaging evaluation to determine the changes in observation indicators and efficacy endpoints in clinical trials, especially whether PFS can bring the final OS benefit. [28-30] The imRECIST criteria use a single-diameter measurement method, and the way to select target lesions and new target lesions is consistent with the RECIST v1.1 criteria, with the same handling of new lesions as that in irRC. However, the current clinical data on imRECIST criteria are not

perfect.

Table 1 shows a detailed comparison of RECIST v1.1, irRC, irRECIST, iRECIST, and imRECIST criteria.

2.2 Evaluation criteria for hyperprogression disease

The above immune-related evaluation criteria are mainly formulated for the identification of pseudoprogression. HPD is a true progression. It occurs not only in the course of immunotherapy but also with IFNα in the treatment of advanced melanoma and anti-CD20 monoclonal antibody rituximab in the treatment of multiple myeloma and diffuse large B-cell tumors, etc. [31-32] Studies have shown that small-molecule tyrosine kinase inhibitors treat a variety of solid tumors. [33-35] Rapid disease progression and deterioration occurred with bevacizumab in the adjuvant treatment of colorectal cancer. [36] The reported incidence of HPD ranges from 6% to 29% for different solid tumors. Therefore, some people think that the occurrence of HPD is a natural pattern of accelerated disease progression due to improper treatment or disease progression due to ineffective treatment. [8] Perhaps because of the high incidence, there is a lack of relevant large prospective randomized controlled studies to confirm it. Therefore, the definition of HPD is currently controversial, and there is no unified evaluation method.

Some small sample size studies based on clinical practice have proposed several main evaluation methods for HPD, all of which are based on RECIST criteria, which have proved that they can effectively identify HPD and timely adjust the medication regimen to achieve certain clinical benefits. [8,10,37] In the clinical study of CHAMPIAT et al., [30] the tumor growth rate (TGR) ≥2 was defined as HPD. KATO et al. [10] defined HPD as TTF<2 months, imaging showing > 2-fold increase of tumor burden compared with that before immunotherapy or tumor growth rate >2. Another group took NSCLC as an example and defined hyperprogression as an increase in ΔTGR > 1.5. [37] Recently, it has been proposed to develop a more reasonable dual time point assessment criteria based on iRECIST criteria.

Table 1 Comparison of immune response evaluation criteria RECIST v1.1 irRC, irRECIST, and imRECIST

Test items	RECIST v1.1[9]	irRC[4]	irRECIST[6]	iRECIST[6]	imRECIST[4]
For measured lesions	Solid tumor lesion: ≥10 mm long diameter, lymph nodes (LN): ≥15 mm short diameter, chest LN: ≥10 mm short diameter)	Lesion ≥5 mm × 5 mm	Same as RECIST v1.1 criteria	Same as RECIST v1.1 criteria	Same as RECIST v1.1 criteria
Number of target lesions	Up to 2 per organ, 5 in total	Up to 5 per organ, a total of	Same as RECIST v1.1	Same as RECIST v1.1 criteria	H RECIST v1.1 criteria

Table 1 Comparison of immune response evaluation criteria RECIST v1.1 irRC, irRECIST, and imRECIST

		10 visceral parenchymal lesions, 5 skin lesions	criteria		
Tumor Burden (TB)	Progression is immediately defined as the sum of the largest diameters of all target lesions using single-diameter measurement	Sum of product of perpendicular diameters of all target lesions using dual diameter measurement is not immediately considered as PD, measurable NL is included in TTB	Same as RECIST v1.1 criteria	Same as RECIST v1.1 criteria	M RECIST v1.1 criteria
New Lesions			Same as irRC criteria	Initial evaluation of iUPD	Same as irRC criteria
Disease progression (PD)	TB increase from baseline by ≥20% or ≥5 mm, equivocal progression of non-target lesions, the occurrence of new lesions	TB increase from baseline by ≥25% (based on measurable target lesions	TB increase from baseline by ≥20% (based on measurable target lesions)	TB increase from baseline by ≥20%; increase in the sum of new target lesions by ≥5 mm: enlargement of new non-target lesions; occurrence of new lesions at the time of confirmation of PD	TB increase from baseline by ≥20% (based on measurable target lesions)
Confirmation of PD	not required	4-12 weeks after starting treatment	4-12 weeks after starting treatment	4-8 weeks after starting treatment	4-8 weeks after starting treatment

3. PET/CT-based immune response evaluation criteria

It is well known that most tumors are highly metabolic tissues. Therefore, it is one-sided to use conventional morphological imaging as the basis for evaluation. In 1999, the European Organization for Research and Treatment of Cancer (EORTC) firstly proposed the metabolism-related anti-tumor treatment efficacy evaluation criteria. [38] In 2009, WAHL et al. [39] proposed the PET/CT response evaluation criteria in solid tumors (PET response criteria in solid tumors, PERCIST) based on EORTC, which first introduced the concepts of standardized uptake value lean (SUL) of imaging agents in local tissues with few body mass requirements and the peak of SUL (SULpeak) in the hot spot lesion area. Taking into account the unconventional responses occurring in the course of immunotherapy and the proposal of irRC criteria, in 2017, ANWAR et al. [40] proposed the first PET/CT-based immunotherapy response evaluation criteria (PET response evaluation criteria for immunotherapy, PERCIMT). They confirmed through the evaluation of 41 patients with advanced melanoma treated with ipilimumab that, compared with the EORTC criteria, the PERCIMT criteria had higher sensitivity. Analyses show that PET/CT can better identify small

lesions that conventional morphological imaging examinations cannot find. The patient's response to treatment is related to changes in the number of lesions, rather than changes in SUV values. However, the PERCIMT criteria directly define new lesions as progression metabolic disease (PMD), which is considered inconsistent with the response pattern of immunotherapy. In the same year, CHO et al. [41] combined the PERCIST and RECIST criteria, and explored the accuracy of PET/CT in evaluating the response at different time points, and formulated the PET/CT criteria for early prediction (treatment days 21-28) of response to ICl therapy (PEPRIT), which confirmed the specificity, sensitivity and accuracy as 93%, 100% and 95% in 20 patients with melanoma treated with ICls. With the emergence of the iRECIST criteria, the immune PET response criteria in solid tumors (iPERCIST) [42] were proposed, which are based on the PERCIST criteria and are similar to the iRECIST criteria, and define the initial evaluation of PMD by the PERCIST criteria as UPMD (uncertain progression metabolic disease) that should be re-evaluated within 4 to 8 weeks. GOLDFARD et al. [42] used the iPERCIST criteria to evaluate 28 NSCLC patients treated with novilumab. Progression was confirmed before ICIs treatment (SCAN-1), 2 months after the start of treatment (SCAN-2), and 4-8 weeks after SCAN-2 (SCAN-3), and 3 or more PET/CT examinations were performed. SCAN-3 is required if it is evaluated as UPMD at the time of the SCAN-2 examination. Those who are determined as progression (CPMD) at SCAN-3 are called non-responders, while those with partial response (PMR) or stable disease (SMD) are called responders. The treatment response rate calculated by this evaluation method is twice higher than that calculated by the iRECIST criteria. There is a significant difference in the prognosis between responders and non-responders, with OS of 19.9 vs 3.6 months, respectively, which suggests that the iPERCIST criteria may have more advantages in evaluating immune-related response. However, the inflammatory response induced by immunotherapy may also lead to a temporary increase in metabolic-related indicators such as SUV, which can be misjudged as pseudoprogression, and the time to the best response based on PET has yet to be verified. [41, 43] Although PET/CT-based evaluation methods are more effective in capturing delayed responses than conventional imaging, [6] the evaluation criteria for PET/CT are mostly derived from retrospective studies of small sample size, so a prospective study of large sample size is required to verify the above criteria.

In addition, PET/CT examinations are not popular, and the complicated examination process limits the further development of relevant criteria. Table 2 shows a detailed comparison of PER- CIST, PERCIMT, and iPERCIST criteria.

Table 2 Comparison of PET/CT-based immune response evaluation criteria PERCIST, PERCIMT and IPERCIST

Measurement	PERCIST[25]	PERCIMT[26]	iPERCIST[26]
Complete response (CMR)	Disappearance of all lesions with high FDG uptake	Disappearance of all lesions with high FDG uptake	Same as PERSIST criteria
Partial response (PMR)	Reduction in FDG SULpeak of target lesions by ≥30%	Disappearance of all lesions with high FDG uptake	Same as PERSIST criteria
Stable disease (SMD)	Except CR, PR, PD	Except CR, PR, PD	Except CR, PR, PD
Progression disease (PMD)	Decrease in PDG SULpeak from baseline by ≥30% or occurrence of new lesions with 18-FDG uptake	Occurrence of 2 or more new lesions > 1 cm or occurrence of 4 or more new lesions < 1 cm	Decrease in FDG SULpeak from baseline by ≥30% or occurrence of new lesions with 18-FDG uptake (UPMD) that need to be reconfirmed after 4-8 weeks (CPMD)

SUL: Standardized uptake value lean of imaging agents in local tissues with few body mass requirements

4 Conclusion

It must be admitted that immunotherapy has indeed improved the prognosis of certain solid tumors, provided a new alternative treatment for the treatment of solid tumors, and also allowed people to see the possibility of turning malignant tumors into chronic diseases or even cures; however, these must be based on accurate clinical response evaluation. Due to unconventional response patterns such as pseudoprogression, delayed response, and HPD in the course of immunotherapy, after the PD as evaluated by the traditional solid tumor response evaluation criteria, the drug is no longer discontinued in a rash manner. Clinical studies have shown [44] that continuing treatment after PD can bring about a 56% response rate and improve the overall survival of patients to a certain extent. At present, the response evaluation criteria widely used in clinical practice are still the classic RECIST v1.1 criteria. On the one hand, the incidence of unconventional reaction patterns is low, and most data show that the incidences of pseudoprogression and hyperprogression are less than 10%, so progressions under most circumstances are true progression. [7] On the other hand, the current various immunotherapy-related clinical response evaluation criteria are still in the research status and need to be further confirmed by prospective randomized controlled clinical trials. The immune-related response evaluation criteria need to be updated and verified, but the relevant biomarkers need to be further developed to more accurately and quickly screen the most suitable population for immunotherapy and bring the best benefits to patients. It can be said that the improvement of immunotherapy response evaluation indicators and the development of immunotherapy complement each other and promote each other. When both are perfect, it is time to overcome cancer completely.

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Appendix 6: Excerpts from Response Evaluation Criteria in Solid Tumors (RECIST 1.1)

(Response Evaluation Criteria in Solid Tumors, RECIST1.1)

1. Background

1.1 History of RECIST criteria

Assessment of the change in tumour burden is an important feature of the clinical evaluation of cancer therapeutics. Both tumour shrinkage (objective response) and time to the development of disease progression are important endpoints in cancer clinical trials. The use of tumour regression as the endpoint for phase II trials screening new agents for evidence of anti-tumour effect is supported by years of evidence suggesting that, for many solid tumours, agents which produce tumour shrinkage in a proportion of patients have a reasonable (albeit imperfect) chance of subsequently demonstrating an improvement in overall survival or other time to event measures in randomised phase III studies (re- viewed in [1–4]). At the current time objective response carries with it a body of evidence greater than for any other biomarker supporting its utility as a measure of promising treatment effect in phase II screening trials. Furthermore, at both the phase II and phase III stage of drug development, clinical trials in advanced disease settings are increasingly utilising time to progression (or progression-free survival) as an endpoint upon which efficacy conclusions are drawn, which is also based on anatomical measurement of tumour size.

However, both of these tumour endpoints, objective response and time to disease progression, are useful only if based on widely accepted and readily applied standard criteria based on anatomical tumour burden. In 1981 the World Health Organisation (WHO) first published tumour response criteria, mainly for use in trials where tumour response was the primary endpoint. The WHO criteria introduced the concept of an overall assessment of tumour burden by summing the products of bidimensional lesion measurements and determined response to therapy by evaluation of change from baseline while on treatment.[5] However, in the decades that followed their publication, cooperative groups and pharmaceutical companies that used the WHO criteria often ‘modified’ them to accommodate new technologies or to address areas that were unclear in the original document. This led to confusion in interpretation of trial results[6] and in fact, the application of varying response criteria was shown to lead to very different conclusions about the efficacy of the same regimen.[7] In response to these problems, an International Working Party was formed in the mid 1990s to standardise and simplify response criteria. New criteria, known as RECIST (Response Evaluation Criteria in Solid Tumours), were published in 2000.[8] Key features of the original RECIST include definitions of minimum size of measurable lesions, instructions on how many lesions to follow (up to 10; a maximum five per organ site), and the use of unidimensional,

rather than bidimensional, measures for overall evaluation of tumour burden. These criteria have subsequently been widely adopted by academic institutions, cooperative groups, and industry for trials where the primary endpoints are objective response or progression. In addition, regulatory authorities accept RECIST as an appropriate guideline for these assessments.

1.2 Why update RECIST?

Since RECIST was published in 2000, many investigators have confirmed in prospective analyses the validity of substituting unidimensional for bidimensional (and even three-dimensional)-based criteria (reviewed in [9]). With rare exceptions (e.g. mesothelioma), the use of unidimensional criteria seems to perform well in solid tumour phase II studies.

However, a number of questions and issues have arisen which merit answers and further clarity. Amongst these are whether fewer than 10 lesions can be assessed without affecting the overall assigned response for patients (or the conclusion about activity in trials); how to apply RECIST in randomised phase III trials where progression, not response, is the primary endpoint particularly if not all patients have measurable disease; whether or how to utilise newer imaging technologies such as FDG-PET and MRI; how to handle assessment of lymph nodes; whether response confirmation is truly needed; and, not least, the applicability of RECIST in trials of targeted non-cytotoxic drugs. This revision of the RECIST guidelines includes updates that touch on all these points.

1.3. Process of RECIST 1.1 development

The RECIST Working Group, consisting of clinicians with expertise in early drug development from academic research organisations, government and industry, together with imaging specialists and statisticians, has met regularly to set the agenda for an update to RECIST, determine the evidence needed to justify the various changes made, and to review emerging evidence. A critical aspect of the revision process was to create a database of prospectively documented solid tumour measurement data obtained from industry and academic group trials. This database, assembled at the EORTC Data Centre under the leadership of Jan Bogaerts and Patrick Therasse (co-authors of this guideline), consists of >6500 patients with >18,000 target lesions and was utilised to investigate the impact of a variety of questions (e.g. number of target lesions required, the need for response confirmation, and lymph node measurement rules) on response and progression-free survival outcomes. The results of this work, which after evaluation by the RECIST Working Group led to most of the changes in this revised guideline, are reported in detail in a separate paper in this special issue.[10] Larry Schwartz and Robert Ford (also co-authors of this guideline) also provided key databases from which inferences have been made that inform these revisions.[11]

The publication of this revised guideline is believed to be timely since it incorporates changes to

simplify, optimise and standardise the assessment of tumour burden in clinical trials. A summary of key changes is found in Appendix I. Be- cause the fundamental approach to assessment remains grounded in the anatomical, rather than functional, assessment of disease, we have elected to name this version RECIST 1.1, rather than 2.0.

1.4. What about volumetric or functional assessment?

This raises the question, frequently posed, about whether it is 'time' to move from anatomic unidimensional assessment of tumour burden to either volumetric anatomical assessment *or* to functional assessment (e.g. dynamic contrast enhanced MRI or CT or (18)F-fluorodeoxyglucose positron emission tomographic (FDG-PET) techniques assessing tumour metabolism). As can be seen, the Working Group and particularly those involved in imaging research, did not believe that there is at present sufficient standardisation and widespread avail- ability to recommend adoption of these alternative assessment methods. The only exception to this is in the use of FDG-PET imaging as an adjunct to determination of progression, as described later in this guideline. As detailed in paper in this special issue[12], we believe that the use of these promising newer approaches (which could either *add to* or *substitute for* anatomical assessment as described in RECIST) requires appropriate and rigorous clinical validation studies. This paper by Sargent et al. illustrates the type of data that will be needed to be able to define 'endpoints' for these modalities and how to determine where and when such criteria/modalities can be used to improve the reliability with which truly active new agents are identified and truly inactive new agents are discarded in comparison to RECIST criteria in phase II screening trials. The RECIST Working Group looks forward to such data emerging in the next few years to allow the appropriate changes to the next iteration of the RECIST criteria.

2. Purpose of this guideline

This guideline describes a standard approach to solid tumour measurement and definitions for objective assessment of change in tumour size for use in adult and paediatric cancer clinical trials. It is expected these criteria will be useful in all trials where objective response is the primary study endpoint, as well as in trials where assessment of stable disease, tumour progression or time to progression analyses are under- taken, since all of these outcome measures are based on an assessment of anatomical tumour burden and its change on study. There are no assumptions in this paper about the pro- portion of patients meeting the criteria for any of these end-points which will signal that an agent or treatment regimen is active: those definitions are dependent on type of cancer in which a trial is being undertaken and the specific agent(s) under study. Protocols must include appropriate statistical sections which define the efficacy parameters upon which the trial sample size and decision criteria are based. In addition to providing definitions and criteria for

assessment of tumour response, this guideline also makes recommendations regarding standard reporting of the results of trials that utilise tumour response as an endpoint.

While these guidelines may be applied in malignant brain tumour studies, there are also separate criteria published for response assessment in that setting.[13] This guideline is *not* in- tended for use for studies of malignant lymphoma since international guidelines for response assessment in lymphoma are published separately.[14]

Finally, many oncologists in their daily clinical practice follow their patients' malignant disease by means of repeated imaging studies and make decisions about continued therapy on the basis of both objective and symptomatic criteria. It is not intended that these RECIST guidelines play a role in that decision making, except if determined appropriate by the treating oncologist.

3. Measurability of tumour at baseline

3.1. Definitions

At baseline, tumour lesions/lymph nodes will be categorised measurable or non-measurable as follows:

3.1.1. Measurable

Tumour lesions: Must be accurately measured in at least one dimension (*longest* diameter in the plane of measurement is to be recorded) with a *minimum* size of:

- 10 mm by CT scan (CT scan slice thickness no greater than 5 mm; see Appendix II on imaging guidance).
- 10 mm caliper measurement by clinical exam (lesions which cannot be accurately measured with calipers should be recorded as non-measurable).
- 20 mm by chest X-ray.

Malignant lymph nodes: To be considered pathologically enlarged *and* measurable, a lymph node must be P15 mm in *short* axis when assessed by CT scan (CT scan slice thickness recommended to be no greater than 5 mm). At baseline and in follow-up, only the *short* axis will be measured and followed (see Schwartz et al. in this Special Issue15). See also notes below on 'Baseline documentation of target and non-target lesions' for information on lymph node measurement.

3.1.2. Non-measurable

All other lesions, including small lesions (longest diameter <10 mm or pathological lymph nodes with ≥10 to <15 mm short axis) as well as truly non-measurable lesions. Lesions considered truly non-measurable include: leptomeningeal disease, ascites, pleural or pericardial effusion, inflammatory breast disease, lymphangitic involvement of skin or lung, abdominal masses/abdominal organomegaly identified by physical exam that is not measurable by reproducible imaging techniques.

3.1.3. Special considerations regarding lesion measurability

Bone lesions, cystic lesions, and lesions previously treated with local therapy require particular comment:

Bone lesions:.

- Bone scan, PET scan or plain films are not considered adequate imaging techniques to measure bone lesions. How- ever, these techniques can be used to confirm the presence or disappearance of bone lesions.
- Lytic bone lesions or mixed lytic-blastic lesions, with *identifiable soft tissue components*, that can be evaluated by cross sectional imaging techniques such as CT or MRI can be considered as measurable lesions if the *soft tissue component* meets the definition of measurability described above.
- Blastic bone lesions are non-measurable.

Cystic lesions:.

- Lesions that meet the criteria for radiographically defined simple cysts should not be considered as malignant lesions (neither measurable nor non-measurable) since they are, by definition, simple cysts.
- 'Cystic lesions' thought to represent cystic metastases can be considered as measurable lesions, if they meet the definition of measurability described above. However, if non- cystic lesions are present in the same patient, these are preferred for selection as target lesions.

Lesions with prior local treatment:

- Tumour lesions situated in a previously irradiated area, or in an area subjected to other loco-regional therapy, are usually not considered measurable unless there has been demonstrated progression in the lesion. Study protocols should detail the conditions under which such lesions would be considered measurable.

3.2. Specifications by methods of measurements

3.2.1. Measurement of lesions

All measurements should be recorded in metric notation, using calipers if clinically assessed. All baseline evaluations should be performed as close as possible to the treatment start and never more than 4 weeks before the beginning of the treatment.

3.2.2. Method of assessment

The same method of assessment and the same technique should be used to characterise each identified and reported lesion at baseline and during follow-up. Imaging based evaluation should always be done rather than clinical examination unless the lesion(s) being followed cannot be imaged but are assessable by clinical exam.

Clinical lesions: Clinical lesions will only be considered measurable when they are superficial and P10 mm diameter as assessed using calipers (e.g. skin nodules). For the case of skin lesions, documentation by colour photography including a ruler to estimate the size of the lesion is suggested. As noted above, when lesions can be evaluated by both clinical exam and imaging, imaging evaluation should be undertaken since it is more objective and may also be reviewed at the end of the study.

Chest X-ray: Chest CT is preferred over chest X-ray, particularly when progression is an important endpoint, since CT is more sensitive than X-ray, particularly in identifying new lesions. However, lesions on chest X-ray may be considered measurable if they are clearly defined and surrounded by aerated lung. See Appendix II for more details.

CT, MRI: CT is the best currently available and reproducible method to measure lesions selected for response assessment. This guideline has defined measurability of lesions on CT scan based on the assumption that CT slice thickness is 5 mm or less. As is described in Appendix II, when CT scans have slice thickness greater than 5 mm, the minimum size for a measurable lesion should be twice the slice thickness. MRI is also acceptable in certain situations (e.g. for body scans). More details concerning the use of both CT and MRI for assessment of objective tumour response evaluation are provided in Appendix II.

Ultrasound: Ultrasound is not useful in assessment of lesion size and should not be used as a method of measurement. Ultrasound examinations cannot be reproduced in their entirety for independent review at a later date and, because they are operator dependent, it cannot be guaranteed that the same technique and measurements will be taken from one assessment to the next (described in greater detail in Appendix II). If new lesions are identified by ultrasound in the course of the study, confirmation by CT or MRI is ad- vised. If there is concern about radiation exposure at CT, MRI may be used instead of CT in selected instances.

Endoscopy, laparoscopy: The utilisation of these techniques for objective tumour evaluation is not advised. However, they can be useful to confirm complete pathological response when biopsies are obtained or to determine relapse in trials where recurrence following complete response or surgical resection is an endpoint.

Tumour markers: Tumour markers *alone* cannot be used to assess *objective* tumour response. If markers are initially above the upper normal limit, however, they must normalise for a patient to be considered in complete response. Because tumour markers are disease specific, instructions for their measurement should be incorporated into protocols on a disease specific basis. Specific guidelines for both CA-125 response (in recurrent ovarian cancer) and PSA response (in recurrent prostate cancer), have been published.[16–18] In addition, the Gynecologic Cancer Intergroup has

developed CA125 progression criteria which are to be integrated with objective tumour assessment for use in first-line trials in ovarian cancer.[19]

Cytology, histology: These techniques can be used to differentiate between PR and CR in rare cases if required by protocol (for example, residual lesions in tumour types such as germ cell tumours, where known residual benign tumours can re- main). When effusions are known to be a potential adverse effect of treatment (e.g. with certain taxane compounds or angiogenesis inhibitors), the cytological confirmation of the neoplastic origin of any effusion that appears or worsens during treatment can be considered if the measurable tumour has met criteria for response or stable disease in order to differentiate between response (or stable disease) and progressive disease.

4. Tumour response evaluation

4.1. Assessment of overall tumour burden and measurable disease

To assess objective response or future progression, it is necessary to estimate the *overall tumour burden at baseline* and use this as a comparator for subsequent measurements. Only patients with measurable disease at baseline should be included in protocols where objective tumour response is the primary endpoint. Measurable disease is defined by the presence of at least one measurable lesion (as detailed above in Section 3). In studies where the primary endpoint is tumour progression (either time to progression or proportion with progression at a fixed date), the protocol must specify if entry is restricted to those with measurable disease or whether patients having non-measurable disease only are also eligible.

4.2. Baseline documentation of 'target' and 'non-target' lesions

When more than one measurable lesion is present at baseline all lesions up to a maximum of five lesions total (and a maximum of two lesions per organ) representative of all involved organs should be identified as *target lesions* and will be re- corded and measured at baseline (this means in instances where patients have only one or two organ sites involved a *maximum* of two and four lesions respectively will be re- corded). For evidence to support the selection of only five tar- get lesions, see analyses on a large prospective database in the article by Bogaerts et al.[10].

Target lesions should be selected on the basis of their size (lesions with the longest diameter), be representative of all involved organs, but in addition should be those that lend themselves to *reproducible repeated measurements*. It may be the case that, on occasion, the largest lesion does not lend it- self to reproducible measurement in which circumstance the next largest lesion which can be measured reproducibly should be selected. To illustrate this point see the example in Fig. 3 of Appendix II.

Lymph nodes merit special mention since they are normal anatomical structures which may be

visible by imaging even if not involved by tumour. As noted in Section 3, pathological nodes which are defined as measurable and may be identified as target lesions must meet the criterion of a short axis of ≥15 mm by CT scan. Only the *short* axis of these nodes will contribute to the baseline sum. The short axis of the node is the diameter normally used by radiologists to judge if a node is involved by solid tumour. Nodal size is normally reported as two dimensions in the plane in which the image is obtained (for CT scan this is almost always the axial plane; for MRI the plane of acquisition may be axial, saggital or coronal). The smaller of these measures is the short axis. For example, an abdominal node which is reported as being 20 mm × 30 mm has a short axis of 20 mm and qualifies as a malignant, measurable node. In this example, 20 mm should be recorded as the node measurement (See also the example in Fig. 4 in Appendix II). All other pathological nodes (those with short axis ≥10 mm but <15 mm) should be considered non-target lesions. Nodes that have a short axis <10 mm are considered non-pathological and should not be recorded or followed. A *sum of the diameters* (longest for non-nodal lesions, short axis for nodal lesions) for all target lesions will be calculated and reported as the *baseline sum diameters*. If lymph nodes are to be included in the sum, then as noted above, only the *short* axis is added into the sum. The baseline sum diameters will be used as reference to further characterise any objective tumour regression in the measurable dimension of the disease.

All other lesions (or sites of disease) including pathological lymph nodes should be identified as *non-target lesions* and should also be recorded at baseline. Measurements are not required and these lesions should be followed as 'present', 'absent', or in rare cases 'unequivocal progression' (more details to follow). In addition, it is possible to record multiple non- target lesions involving the same organ as a single item on the case record form (e.g. 'multiple enlarged pelvic lymph nodes' or 'multiple liver metastases').

4.3. Response criteria

This section provides the definitions of the criteria used to determine objective tumour response for target lesions.

4.3.1. Evaluation of target lesions

Complete Response (CR): Disappearance of all target lesions. Any pathological lymph nodes (whether target or non-target) must have reduction in short axis to <10 mm.

Partial Response (PR): At least a 30% decrease in the sum of diameters of target lesions, taking as reference the baseline sum diameters.

Progressive Disease (PD): At least a 20% increase in the sum of diameters of target lesions, taking as reference the *smallest sum on study* (this includes the baseline sum if that is the smallest on study). In addition to the relative increase of 20%, the sum must also demonstrate an absolute increase of at least 5 mm. (*Note:* the appearance of one or more new lesions is also considered progression).

Stable Disease (SD): Neither sufficient shrinkage to qualify for PR nor sufficient increase to qualify for PD, taking as reference the smallest sum diameters while on study.

4.3.2. Special notes on the assessment of target lesions Lymph nodes.

Lymph nodes identified as target lesions should always have the actual short axis measurement recorded (measured in the same anatomical plane as the baseline examination), even if the nodes regress to below 10 mm on study. This means that when lymph nodes are included as target lesions, the 'sum' of lesions may not be zero even if complete response criteria are met, since a normal lymph node is defined as having a short axis of <10 mm. Case report forms or other data collection methods may therefore be designed to have target nodal lesions recorded in a separate section where, in order to qualify for CR, each node must achieve a short axis <10 mm. For PR, SD and PD, the actual short axis measurement of the nodes is to be included in the sum of target lesions.

Target lesions that become 'too small to measure'. While on study, all lesions (nodal and non-nodal) recorded at baseline should have their actual measurements recorded at each sub- sequent evaluation, even when very small (e.g. 2 mm). How- ever, sometimes lesions or lymph nodes which are recorded as target lesions at baseline become so faint on CT scan that the radiologist may not feel comfortable assigning an exact measure and may report them as being 'too small to measure'. When this occurs it is important that a value be recorded on the case report form. If it is the opinion of the radiologist that the lesion has likely disappeared, the measurement should be recorded as 0 mm. If the lesion is believed to be present and is faintly seen but too small to measure, a default value of 5 mm should be assigned (*Note:* It is less likely that this rule will be used for lymph nodes since they usually have a definable size when normal and are frequently surrounded by fat such as in the retroperitoneum; however, if a lymph node is believed to be present and is faintly seen but too small to measure, a de- fault value of 5 mm should be assigned in this circumstance as well). This default value is derived from the 5 mm CT slice thickness (but should not be changed with varying CT slice thickness). The measurement of these lesions is potentially non-reproducible, therefore providing this default value will prevent false responses or progressions based upon measure- ment error. To reiterate, however, if the radiologist *is* able to provide an actual measure, that should be recorded, even if it is below 5 mm.

Lesions that split or coalesce on treatment. As noted in Appendix II, when non-nodal lesions

'fragment', the longest diameters of the fragmented portions should be added together to calculate the target lesion sum. Similarly, as lesions coalesce, a plane between them may be maintained that would aid in obtaining maximal diameter measurements of each individual lesion. If the lesions have truly coalesced such that they are no longer separable, the vector of the longest diameter in this instance should be the maximal longest diameter for the 'coalesced lesion'.

4.3.3. Evaluation of non-target lesions

Thissection provides the definitionsof the criteria used to deter- mine the tumour response for the group of non-target lesions. While some non-target lesions may actually be measurable, they need not be measured and instead should be assessed only *qualitatively* at the time points specified in the protocol.

Complete Response (CR): Disappearance of all non-target lesions and normalisation of tumour marker level. All lymph nodes must be non-pathological in size (<10 mm short axis).

Non-CR/Non-PD: Persistence of one or more non-target lesion(s) and/or maintenance of tumour marker level above the normal limits.

Progressive Disease (PD): *Unequivocal progression* (see comments below) of existing non-target lesions. (*Note:* the appearance of one or more new lesions is also considered progression).

4.3.4. Special notes on assessment of progression of non- target disease

The concept of progression of non-target disease requires additional explanation as follows:

When the patient also has measurable disease. In this setting, to achieve 'unequivocal progression' on the basis of the non-target disease, there must be an overall level of substantial worsening in non-target disease such that, even in presence of SD or PR in target disease, the overall tumour burden has increased sufficiently to merit discontinuation of therapy (see examples in Appendix II and further details below). A modest 'increase' in the size of one or more non-tar- get lesions is usually not sufficient to quality for unequivocal progression status. The designation of overall progression *solely* on the basis of change in non-target disease in the face of SD or PR of target disease will therefore be extremely rare.

When the patient has only non-measurable disease. This circumstance arises in some phase III trials when it is not a criterion of study entry to have measurable disease. The same general concepts apply here as noted above, however, in this instance there is no measurable disease assessment to factor into the interpretation of an increase in non-measurable disease burden. Because worsening in non-target disease cannot be easily quantified (by definition: if all lesions are truly non-measurable) a useful test that can be applied when assessing patients for unequivocal progression is to consider if the increase in overall disease burden based on the change in non-measurable disease is comparable in magnitude to the increase that would berequired todeclare

PD for measurabledisease: i.e. anincrease in tumour burden representing an additional 73% increase in ‘volume’ (which is equivalent to a 20% increase diameter in a measurable lesion). Examples include an increase in a pleural effusion from ‘trace’ to ‘large’, an increase in lymphangitic disease from localised to widespread, or may be described in protocols as ‘sufficient to require a change in therapy’. Some illustrative examples are shown in Figs. 5 and 6 in Appendix II. If ‘unequivocal progression’ is seen, the patient should be considered to have had overall PD at that point. While it would be ideal to have objective criteria to apply to non-measurable disease, the very nature of that disease makes it impossible to do so, therefore the increase must be substantial.

4.3.5. New lesions

The appearance of new malignant lesions denotes disease progression; therefore, some comments on detection of new lesions are important. There are no specific criteria for the identification of new radiographic lesions; however, the finding of a new lesion should be unequivocal: i.e. not attributable to differences in scanning technique, change in imaging modality or findings thought to represent something other than tumour (for example, some ‘new’ bone lesions may be simply healing or flare of pre-existing lesions). This is particularly important when the patient’s baseline lesions show partial or complete response. For example, necrosis of a liver lesion may be reported on a CT scan report as a ‘new’ cystic lesion, which it is not.

A lesion identified on a follow-up study in an anatomical location that was *not* scanned at baseline is considered a new lesion and will indicate disease progression. An example of this is the patient who has visceral disease at baseline and while on study has a CT or MRI brain ordered which reveals metastases. The patient’s brain metastases are considered to be evidence of PD even if he/she did not have brain imaging at baseline.

If a new lesion is equivocal, for example because of its small size, continued therapy and follow-up evaluation will clarify if it represents truly new disease. If repeat scans con- firm there is definitely a new lesion, then progression should be declared using the date of the initial scan.

While FDG-PET response assessments need additional study, it is sometimes reasonable to incorporate the use of FDG-PET scanning to complement CT scanning in assessment of progression (particularly possible ‘new’ disease). New lesions on the basis of FDG-PET imaging can be identified according to the following algorithm:

a. Negative FDG-PET at baseline, with a positive[1] FDG-PET at follow-up is a sign of PD based on a new lesion.

[1] A ‘positive’ FDG-PET scan lesion means one which is FDG avid with an uptake greater than twice that of the surrounding tissue on the attenuation corrected image.

b. No FDG-PET at baseline and a positive FDG-PET at follow-up:

If the positive FDG-PET at follow-up corresponds to a new site of disease confirmed by CT, this is PD.

If the positive FDG-PET at follow-up is not confirmed as a new site of disease on CT, additional follow-up CT scans are needed to determine if there is truly progression occurring at that site (if so, the date of PD will be the date of the initial abnormal FDG-PET scan).

If the positive FDG-PET at follow-up corresponds to a pre-existing site of disease on CT that is not progressing on the basis of the anatomic images, this is not PD.

4.4. Evaluation of best overall response

The best overall response is the best response recorded from the start of the study treatment until the end of treatment taking into account any requirement for confirmation. On occasion a response may not be documented until after the end of therapy so protocols should be clear if post-treatment assessments are to be considered in determination of best overall response. Protocols must specify how any new therapy introduced before progression will affect best response designation. The patient's best overall response assignment will depend on the findings of both target and non-target disease and will also take into consideration the appearance of new lesions. Furthermore, depending on the nature of the study and the protocol requirements, it may also require confirmatory measurement (see Section 4.6). Specifically, in non-ran-domised trials where response is the primary endpoint, confirmation of PR or CR is needed to deem either one the 'best overall response'. This is described further below.

4.4.1. Time point response

It is assumed that at each protocol specified time point, a re- sponse assessment occurs. Table 1 on the next page provides a summary of the overall response status calculation at each time point for patients who have measurable disease at baseline.

When patients have non-measurable (therefore non-tar- get) disease only, Table 2 is to be used.

4.4.2. Missing assessments and inevaluable designation

When no imaging/measurement is done at all at a particular time point, the patient is not evaluable (NE) at that time point. If only a subset of lesion measurements are made at an assessment, usually the case is also considered NE at that time point, unless a convincing argument can be made that the contribution of the individual missing lesion(s) would not change the assigned time point response. This would be most likely to happen in the case of PD. For example, if a patient had a baseline sum of 50 mm with three measured lesions and at follow-up only two lesions were assessed, but those gave a sum of 80 mm, the patient will have achieved PD status, regardless of

the contribution of the missing lesion.

4.4.3. Best overall response: all time points

The *best overall response* is determined once all the data for the patient is known.

Best response determination in trials where confirmation of complete or partial response IS NOT required: Best response in these trials is defined as the best response across all time points (for example, a patient who has SD at first assessment, PR at second assessment, and PD on last assessment has a best overall response of PR). When SD is believed to be best response, it must also meet the protocol specified minimum time from baseline. If the minimum time is not met when SD is other- wise the best time point response, the patient's best response depends on the subsequent assessments. For example, a patient who has SD at first assessment, PD at second and does not meet minimum duration for SD, will have a best response of PD. The same patient lost to follow-up after the first SD assessment would be considered inevaluable.

Table 1 – Time point response: patients with target (+/– non-target) disease.

Target lesions	Non-target lesions	New lesions	Overall response
CR	CR	No	CR
CR	Non-CR/non-PD	No	PR
CR	Not evaluated	No	PR
PR	Non-PD or not all evaluated	No	PR
SD	Non-PD or not all evaluated	No	SD
Not all evaluated	Non-PD	No	NE
PD	Any	Yes or No	PD
Any	PD	Yes or No	PD
Any	Any	Yes	PD

CR = complete response, PR = partial response, SD = stable disease, PD = progressive disease, and NE = inevaluable.

Table 2 – Time point response: patients with non-target disease only.

Non-target lesions	New lesions	Overall response
CR	No	CR
Non-CR/non-PD	No	Non-CR/non-PDa
Not all evaluated	No	NE
Unequivocal PD	Yes or No	PD
Any	Yes	PD

CR = complete response, PD = progressive disease, and NE = inevaluable.
a 'Non-CR/non-PD' is preferred over 'stable disease' for non-target disease since SD is increasingly used as endpoint for assessment of efficacy in some trials so to assign this category when no lesions can be measured is not advised.

Best response determination in trials where confirmation of complete or partial response IS required: Complete or partial responses may be claimed only if the criteria for each are met at a subsequent time point as specified in the protocol (generally 4 weeks later). In this circumstance, the best overall response can be interpreted as in Table 3.

4.4.4. Special notes on response assessment

When nodal disease is included in the sum of target lesions and the nodes decrease to 'normal' size (<10 mm), they may still have a measurement reported on scans. This measurement should be

recorded even though the nodes are normal in order not to overstate progression should it be based on increase in size of the nodes. As noted earlier, this means that patients with CR may not have a total sum of ‘zero’ on the case report form (CRF).

In trials where confirmation of response is required, repeated ‘NE’ time point assessments may complicate best response determination. The analysis plan for the trial must address how missing data/assessments will be addressed in determination of response and progression. For example, in most trials it is reasonable to consider a patient with time point responses of PR-NE-PR as a confirmed response.

Patients with a global deterioration of health status requiring discontinuation of treatment without objective evidence of disease progression at that time should be reported as ‘symptomatic deterioration’. Every effort should be made to document objective progression even after discontinuation of treatment. Symptomatic deterioration is *not* a descriptor of an objective response: it is a reason for stopping study therapy. The objective response status of such patients is to be determined by evaluation of target and non-target disease as shown in Tables 1–3.

Conditions that define ‘early progression, early death and inevaluability’ are study specific and should be clearly de- scribed in each protocol (depending on treatment duration, treatment periodicity).

In some circumstances it may be difficult to distinguish residual disease from normal tissue. When the evaluation of complete response depends upon this determination, it is recommended that the residual lesion be investigated (fine needle aspirate/biopsy) before assigning a status of complete response. FDG-PET may be used to upgrade a response to a CR in a manner similar to a biopsy in cases where a residual radiographic abnormality is thought to represent fibrosis or scarring. The use of FDG-PET in this circumstance should be prospectively described in the protocol and supported by dis- ease specific medical literature for the indication. However, it must be acknowledged that both approaches may lead to false positive CR due to limitations of FDG-PET and biopsy resolution/sensitivity.

Table 3 – Best overall response when confirmation of CR and PR required.

Overall response First time point	Overall response Subsequent time point	BEST overall response
CR	CR	CR
CR	PR	SD, PD or PR[a]
CR	SD	SD provided minimum criteria for SD duration met, otherwise, PD
CR	PD	SD provided minimum criteria for SD duration met, otherwise, PD
CR	NE	SD provided minimum criteria for SD duration met, otherwise NE
PR	CR	PR
PR	PR	PR
PR	SD	SD
PR	PD	SD provided minimum criteria for SD duration met, otherwise, PD
PR	NE	SD provided minimum criteria for SD duration met, otherwise NE
NE	NE	NE

CR = complete response, PR = partial response, SD = stable disease, PD = progressive disease, and NE = inevaluable.

a If a CR is *truly* met at first time point, then any disease seen at a subsequent time point, even disease meeting PR criteria relative to baseline, makes the disease PD at that point (since disease must have reappeared after CR). Best response would depend on whether minimum duration for SD was met. However, sometimes 'CR' may be claimed when subsequent scans suggest small lesions were likely still present and in fact the patient had PR, not CR at the first time point. Under these circumstances, the original CR should be changed to PR and the best response is PR.

For equivocal findings of progression (e.g. very small and uncertain new lesions; cystic changes or necrosis in existing lesions), treatment may continue until the next scheduled assessment. If at the next scheduled assessment, progression is confirmed, the date of progression should be the earlier date when progression was suspected.

4.5. Frequency of tumour re-evaluation

Frequency of tumour re-evaluation while on treatment should be protocol specific and adapted to the type and schedule of treatment. However, in the context of phase II studies where the beneficial effect of therapy is not known, follow-up every 6–8 weeks (timed to coincide with the end of a cycle) is reasonable. Smaller or greater time intervals than these could be justified in specific regimens or circumstances. The proto- col should specify which organ sites are to be evaluated at baseline (usually those most likely to be involved with meta- static disease for the tumour type under study) and how often evaluations are repeated. Normally, all target and non-target sites are evaluated at each assessment. In selected circumstances certain non-target organs may be evaluated less frequently. For example, bone scans may need to be repeated only when complete response is identified in target disease or when progression in bone is suspected.

After the end of the treatment, the need for repetitive tumour evaluations depends on whether the trial has as a goal the response rate or the time to an event (progression/death). If 'time to an event' (e.g. time to progression, disease-free survival, progression-free survival) is the main endpoint of

the study, then routine scheduled re-evaluation of protocol specified sites of disease is warranted. In randomised comparative trials in particular, the scheduled assessments should be performed as identified on a calendar schedule (for example: every 6–8 weeks on treatment or every 3–4 months after treatment) and should not be affected by delays in therapy, drug holidays or any other events that might lead to imbalance in a treatment arm in the timing of disease assessment.

4.6. Confirmatory measurement/duration of response

4.6.1. Confirmation

In non-randomised trials where response is the primary end- point, confirmation of PR and CR is required to ensure responses identified are not the result of measurement error. This will also permit appropriate interpretation of results in the context of historical data where response has traditionally required confirmation in such trials (see the paper by Bogaerts et al. in this Special Issue10). However, in all other circumstances, i.e. in randomised trials (phase II or III) or studies where stable disease or progression are the primary endpoints, confirmation of response is not required since it will not add value to the interpretation of trial results. However, elimination of the requirement for response confirmation may increase the importance of central review to protect against bias, in particular in studies which are not blinded.

In the case of SD, measurements must have met the SD criteria at least once after study entry at a minimum interval (in general not less than 6–8 weeks) that is defined in the study protocol.

4.6.2. Duration of overall response

The duration of overall response is measured from the time measurement criteria are first met for CR/PR (whichever is first recorded) until the first date that recurrent or progressive dis- ease is objectively documented (taking as reference for progressive disease the smallest measurements recorded on study).

The duration of overall complete response is measured from the time measurement criteria are first met for CR until the first date that recurrent disease is objectively documented.

4.6.3. Duration of stable disease

Stable disease is measured from the start of the treatment (in randomised trials, from date of randomisation) until the criteria for progression are met, taking as reference the *smallest sum on study* (if the baseline sum is the smallest, this is the reference for calculation of PD).

The clinical relevance of the duration of stable disease varies in different studies and diseases. If the proportion of patients achieving stable disease for a minimum period of time is an endpoint of importance in a particular trial, the protocol should specify the minimal time interval required between two measurements for determination of stable disease.

Note: The duration of response and stable disease as well as theprogression-free survival are

influenced by the frequency of follow-up after baseline evaluation. It is not in the scope of this guideline to define a standard follow-up frequency. The frequency should take into account many parameters including disease types and stages, treatment periodicity and standard practice. However, these limitations of the precision of the measured endpoint should be taken into account if comparisons between trials are to be made.

4.7. Progression-free survival/proportion progression-free

4.7.1. Phase II trials

This guideline is focused primarily on the use of objective response endpoints for phase II trials. In some circumstances, ‘response rate’ may not be the optimal method to assess the potential anticancer activity of new agents/regimens. In such cases ‘progression-free survival’ (PFS) or the ‘proportion progression-free’ at landmark time points, might be considered appropriate alternatives to provide an initial signal of biologic effect of new agents. It is clear, however, that in an uncontrolled trial, these measures are subject to criticism since an apparently promising observation may be related to biological factors such as patient selection and not theimpact of theintervention. Thus, phase II screening trials utilising these endpoints are best designed with a randomised control. Exceptions may exist where the behaviour patterns of certain cancers are so consistent (and usually consistently poor), that a non-randomised trial is justifiable (see for example van Glabbeke et al. 20). How- ever, in these cases it will be essential to document with care the basis for estimating the expected PFS or proportion progression-free in the absence of a treatment effect.

Appendix 7: Modified criteria for the evaluation of efficacy in solid tumours (mRECIST)

Modified RECIST (mRECIST) Assessment for Hepatocellular Carcinoma, 2020 version

Target Lesions Assessment in mRECIST	
* Complete response (CR)	All typical intrahepatic targets (arterial enhancement) disappeared, all atypical intrahepatic and extrahepatic targets disappeared, and all pathological lymph nodes had a short diameter of <10 mm.
* Partial response (PR)	The diameter of all typical intrahepatic target lesions (arterial phase enhancement) and the sum of all atypical intrahepatic target lesions and extrahepatic target lesions (including short diameters of lymph node lesions) were reduced by at least 30%, using the sum of the baseline diameters of the target lesions as a reference value.
* Progressive disease (PD)	The sum of the diameters of all typical intrahepatic target lesions (arterial phase enhancement) and all atypical intrahepatic target lesions and extrahepatic target lesions (including short diameters of lymph node lesions) increased by at least 20% in relative terms and by at least 5 mm in absolute terms, using the lowest sum of diameters recorded since baseline as a reference.
* stable disease (SD)	All target lesions did not shrink to PR (using the sum of baseline diameters as a reference value) nor did they grow to PD (using the minimum of the sum of target lesion diameters as a reference value).
no target lesion	At baseline, the target lesion was not identified (no arterial phase enhancement measurable lesion).
non-evaluable (NE)	Failure to complete enhanced CT or enhanced MRI scans, incomplete scans, or failure to evaluate due to poor scan quality at the time point of evaluation of the target lesion.
Not evaluated	No imaging was performed
* Diameters：	Lymph node lesions: shortest axis
	Non-lymph node lesions: maximum diameter

Non-Target Lesions Assessment in mRECIST	
Complete response (CR)	All typical intrahepatic non-target lesions (arterial phase enhancement) disappeared, all atypical intrahepatic non-target lesions and extrahepatic non-target lesions disappeared, and the short diameter of pathological lymph nodes was <10 mm.
Non-CR/Non-PD	One or more typical intrahepatic nontarget lesions (arterial phase enhancement) or atypical intrahepatic nontarget lesions or persistent extrahepatic nontarget lesions.
Progressive disease (PD)	Clear progression of typical intrahepatic non-target lesions (arterial phase enhancement) or clear progression of atypical intrahepatic non-target lesions or extrahepatic non-target lesions.
no non-target lesion	Non-target lesions were not identified at baseline (no arterial phase enhancement of non-target lesions).

non-evaluable (NE)	Failure to complete enhanced CT or enhanced MRI scans, incomplete scans, or failure to evaluate due to poor scan quality at the time point of evaluation of non-target lesions.
Not evaluated	No imaging was performed

Overall Response Assessment in mRECIST: Responses for All Possible Combinations of Tumor Responses in Target and Nontarget Lesions with or without the Appearance of New Lesions			
Target Lesions	Nontarget Lesions	New Lesions	Overall Response
CR	CR	NO	CR
CR	Non-CR/Non-PD	NO	PR
PR	Non-PD	NO	PR
SD	Non-PD	NO	SD
PD	any	yes/no	PD
any	PD	yes/no	PD
any	any	yes	PD
Non-PD	Non-PD	to be confirmed	PD to be confirmed
NA	Surviving target lesions and surviving non-target lesions not identified at baseline		
Not evaluated	No imaging was performed		
Note: Non-evaluable (NE) is defined as results that cannot be evaluated due to failure to complete the required scans, incomplete scans, poor quality scans, or changes in inspection methodology.			

Notes:

Surviving tumour: lesion showing contrast uptake in the arterial phase on dynamic CT or MRI

Surviving target lesion: 1. Enhanced CT or MRI shows intratumoural arterial phase enhancement; 2. Lesion can be classified as RECIST measurable (i.e., the lesion can be accurately measured in at least one dimension of 1 cm or more). 3. Lesion is amenable to accurate and repeated measurements.

Typical intrahepatic target lesion: enhanced CT or enhanced MRI showing an intratumoural (non-marginal) arterial-phase enhancing lesion in the liver with a maximum diameter of ≥1 cm (suitable for accurate and repeated measurement)

Atypical intrahepatic lesions: enhancement CT or enhancement MRI does not show an intratumoral hepatic arterial-phase enhancing lesion, requiring measurement of the entire tumour diameter, including well-differentiated neovascularly immature tumours; steatotic and cystic tumours; invasive poorly differentiated carcinomas; and HCC with borderline-like arterial enhancement;

Lymph nodes: when used as target lesions, hilar lymph nodes were required to have a short-axis diameter of ≥2 cm, and lymph nodes elsewhere should have a short-axis diameter of ≥1.5 cm.

New intrahepatic lesions: a new hepatic lesion ≥1 cm with arterial phase enhancement is declared PD. any new hepatic lesion smaller than 1 cm or any new hepatic lesion of any size that does not show arterial phase enhancement should be considered ambiguous, and progression should only be diagnosed if arterial enhancement of ≥1 cm is seen on a subsequent enhancement scan. If an ill-defined new lesion is later determined to be definite, the time point of progression will be the time point at which the lesion was initially found to be ill-defined.

The maximum total number of target lesions (intra-hepatic plus extra-hepatic) was limited to five, with a maximum of two lesions per organ with these features, with preference given to typical intra-hepatic lesions as target lesions. If measurements of surviving tumour components may be inaccurate and/or inconsistent, the entire tumour diameter should be recorded, as in the case of atypical intrahepatic or extrahepatic target lesions. The sum of the baseline diameters of the target lesions is: the sum of the surviving tumour diameters of the typical intrahepatic target lesions, the sum of the total diameters of the atypical intrahepatic target lesions and non-lymphoidal extrahepatic target lesions, and the sum of the short-axis diameters of the lymphoidal target lesions.

Non-target lesions: tumour necrosis should be considered when assessing the response of typical intrahepatic non-target lesions. Complete resolution of malignant portal vein intra-thrombotic enhancement should be considered equivalent to CR. the appearance or worsening of ascites or pleural effusion during treatment should not be considered a manifestation of PD unless associated with the appearance or definite progression of a peritoneal or thoracic tumour nodule, or when cytology confirms its malignant nature.