

Novel Oncolytic Virus VG161 in Hepatocellular Carcinoma Patients Refractory to Standard Systemic Therapies

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**Novel Oncolytic Virus VG161 in Hepatocellular Carcinoma Patients Refractory
to Standard Systemic Therapies**

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Abstract

For hepatocellular carcinoma (HCC) patients, treatment options are limited after failing second-line therapy. This phase I, multicenter trial evaluates the safety and antitumor efficacy of VG161 in patients with advanced liver cancer. The study included dose escalation using a '3 + 3' design across five dose levels, followed by a dose expansion phase using Simon's two-stage design. Forty-four patients received doses with no dose-limiting toxicity observed. Among the 40 HCC patients, 37 were evaluable. The objective response rate (ORR) was 18.92%, and the disease control rate (DCR) was 64.86%. The median progression-free survival (PFS) was 2.9 months, and overall survival (OS) was 12.4 months. Subgroup analysis showed that among 34 patients who had failed second-line treatment, the ORR was 17.65% and the DCR was 64.71%. The median PFS and OS for this group were 2.8 and 9.4 months, respectively. Patients with prior checkpoint inhibitor treatment (PreCPI) longer than 3 months had a median OS of 17.3 months, compared to 7.4 months for those with PreCPI ≤ 3 m ($p = 0.04$). Additionally, patients receiving systemic therapies after VG161 showed longer survival ($p = 0.03$). Multiple detection techniques revealed that VG161 reshapes the immune microenvironment of HCC, enhancing tumor sensitivity to systemic treatments. An efficacy prediction model based on differentially expressed genes was established, which successfully predicts prolonged OS in study patients ($p < 0.001$) but not in public database patients ($p = 0.17$). Anti-hepatitis B agents were confirmed to not affect the replication and efficacy of VG161. In summary, VG161 demonstrated comparable efficacy to second-line treatments in third-line patients, providing a new option for

63 patients who have failed second-line therapy. It was well-tolerated and showed
64 promising efficacy, extending survival in some subgroups and enhancing post-
65 treatment systemic therapy efficacy, supporting its potential use in combination
66 treatments. ClinicalTrials.gov registration: NCT04806464.

Hepatocellular carcinoma (HCC) remains one of the most life-threatening cancers globally, exhibiting a dire prognosis with an approximate 5-year survival rate of 18%¹. Despite the close correlation between incidence and mortality², advances in targeted drugs and immunotherapies have provided substantial benefits for late-stage HCC patients. Current treatments, including multi-targeted agents like sorafenib, lenvatinib, cabozantinib, and regorafenib, have demonstrated significant clinical benefits³⁻⁶. Sorafenib, in particular, has notably altered the therapeutic landscape for advanced HCC. However, the dose-dependent adverse effects associated with these drugs often necessitate dose reductions or discontinuation, undermining their efficacy^{7,8}.

The integration of checkpoint inhibitors (CPIs) into the therapeutic arsenal has diversified treatment options, with combinations such as bevacizumab with atezolizumab^{9,10} and dual CPI therapies including durvalumab and tremelimumab¹¹ showing promising results. Nevertheless, the therapeutic landscape for HCC remains largely uniform, relying heavily on targeted therapies and CPIs, with minimal variance in mechanisms, thus capping further improvements in treatment outcomes. Consequently, no standard treatments are recommended beyond the second line, highlighting a critical gap for innovative therapies.

Oncolytic viruses (OVs) offer a novel mechanism by targeting cancer cells for selective replication and lysis, thereby stimulating anti-cancer immunity. These viruses not only destroy cancer cells but also release cancer-associated neoantigens, enhancing systemic antitumor immunity¹². The initial trials with JX-594, an oncolytic virus expressing granulocyte-macrophage colony-stimulating factor (GM-CSF),

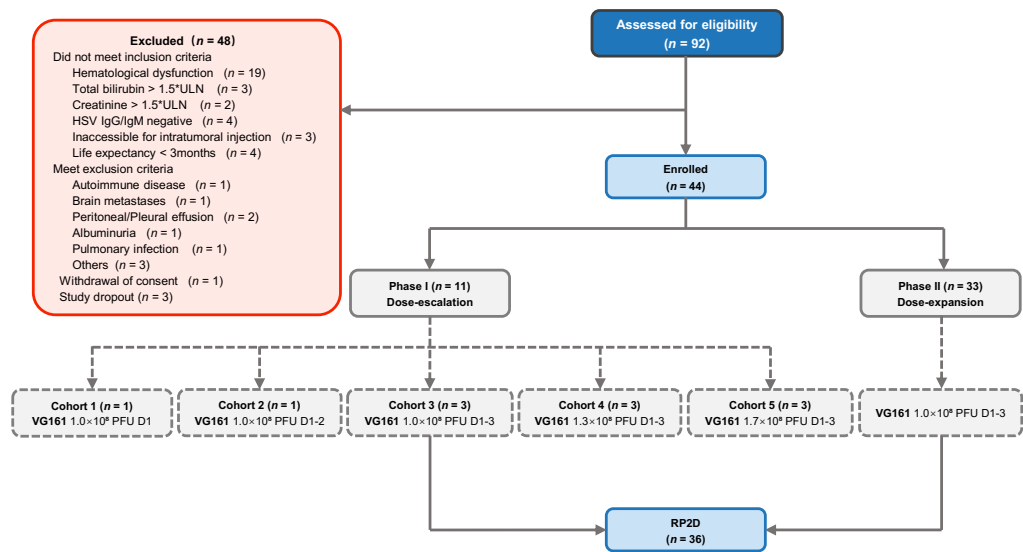
demonstrated safety but failed to show sustained clinical benefits in subsequent studies^{13,14}, possibly due to the dual role of GM-CSF in both stimulating and suppressing immune responses¹⁵⁻¹⁸.

Building on this foundation, we have developed VG161, an oncolytic herpes simplex virus (HSV) modified to express interleukin-12, -15, IL-15 receptor alpha, and a PD-1/PD-L1 blocking fusion protein¹⁹. Preliminary preclinical studies have validated that VG161 effectively remodels the tumor immune microenvironment and induces potent antitumor responses²⁰. This paper reports on a phase I multicenter clinical trial of VG161 in patients with advanced primary liver cancer refractory to standard systemic therapies, detailing its safety, tolerability, pharmacokinetics, and pharmacodynamics, including its impact on the immune landscape and therapeutic efficacy.

Patient enrollment summary

Between April 8, 2021, and November 21, 2023, we enrolled 44 patients across three participating centers. The cohort was divided into dose escalation (11 patients) and dose expansion phases (33 patients) (Extended Data Fig. 1). Table 1 details the patients' baseline characteristics. All participants were included in the safety analysis. Only patients who completed at least one tumor assessment were included in the efficacy analysis. As of the January 12, 2024, data cut-off, the median follow-up period was 7.0 months (range: 1.7-26.3 months).

Table 1 Demographic and baseline characteristics			
	Dose escalation n=11 (%)	Dose expansion n=33 (%)	Total n=44 (%)
Age (years)			
Mean ± SD	59.7 ± 9.7	57.9 ±9.3	58.3 ±9.3
Median	58	59	59
Minimum, maximum	43, 73	39, 72	39, 73
Gender (%)			
Male	9 (81.8)	29 (87.9)	38 (86.4)
Female	2 (18.2)	4 (12.1)	6 (13.6)
Race (%)			
Chinese (Ethnic Han)	11 (100)	32 (97)	43 (97.7)
China (Ethnic minorities)	0 (0)	1 (3)	1 (2.3)
HBV positive	8 (72.7)	26 (78.8)	34 (77.3)
Anti-HBV therapy	6 (54.5)	25 (75.8)	31 (70.5)
HCV positive	0 (0)	0 (0)	0 (0)
ECOG (%)			
0	7 (63.6)	3 (9.1)	10 (22.7)
1	4 (36.4)	30 (90.9)	34 (77.3)
Pathological diagnosis			
HCC	8 (72.7)	32 (97)	40 (90.9)
ICC	3 (27.3)	1 (3)	4 (9.1)
Prior treatments			
Tumor-related surgery	8 (72.7)	25 (75.8)	33 (75)
Radiotherapy	3 (27.3)	8 (24.2)	11 (25)
TACE	6 (54.5)	31 (93.9)	37 (84.1)
Systemic therapy			
Chemotherapy	3 (27.3)	1 (3)	4 (9.1)
Targeted therapy	11 (100)	33 (100)	44 (100)
Immunotherapy	11 (100)	32 (97)	43 (97.7)
Abbreviation: ECOG, eastern cooperative oncology group; HBV, hepatitis B virus; HCV, hepatitis C virus; HCC, hepatocellular carcinoma; ICC, intrahepatic cholangiocarcinoma; TACE, transcatheter arterial chemoembolization.			



Extended Data Fig. 1| Patient enrolment. Of 92 patients who provided informed consent, 44 patients who matched the eligibility criteria were enrolled.

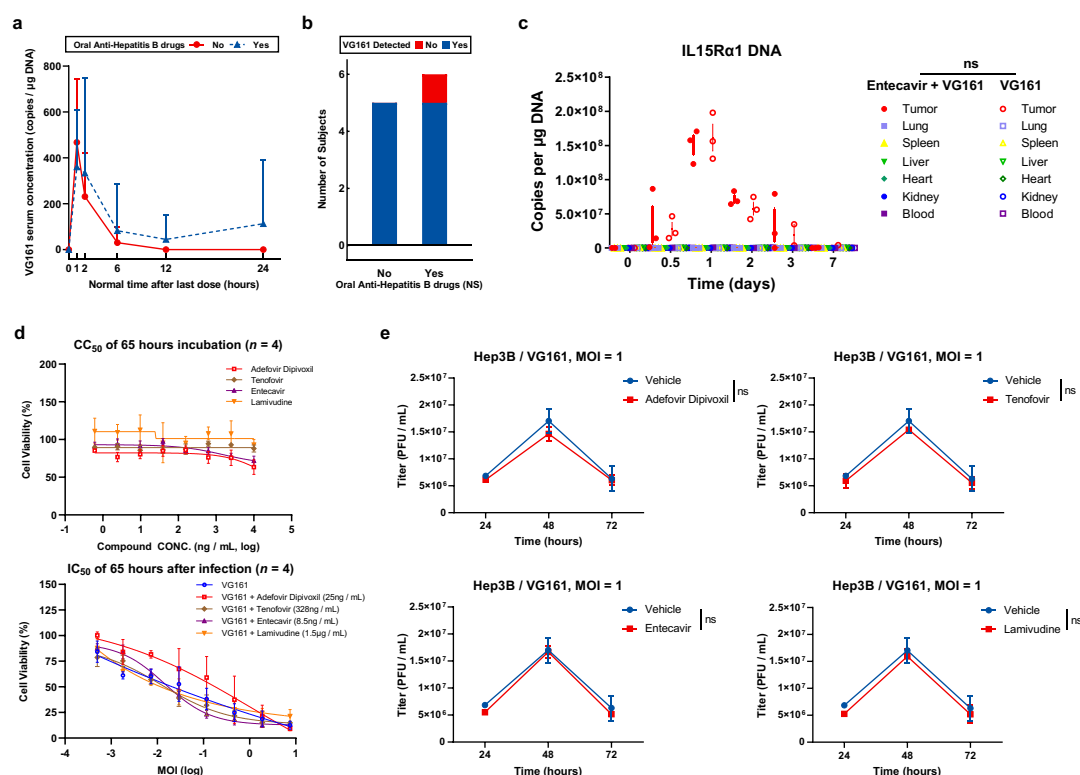
Pharmacokinetic and pharmacodynamic profiles

Patients in the dose escalation phase received intratumoral injections of 1.0×10^8 to 5.0×10^8 PFU VG161. VG161 was detectable in the blood within 24 h post-administration but decreased below detectable levels after 24 h. The dose and VG161 DNA concentration in the blood (range: 156.85-1114.39 copies/ μ g) were not significantly correlated. Viral shedding was not detected in urine, fecal, or saliva post-administration.

As many patients had hepatitis B-induced HCC, we investigated whether oral anti-hepatitis B agents influenced VG161 efficacy. The patients were divided into those taking antiviral agents (n = 6) and those not (n = 5), comprising eight patients with HCC and three patients with ICC. The detectable VG161 DNA concentrations in the blood were not significantly different between the two groups (Extended Data Fig. 2a, 2b).

The subcutaneously transplanted mouse HCC tumor model received intratumoral

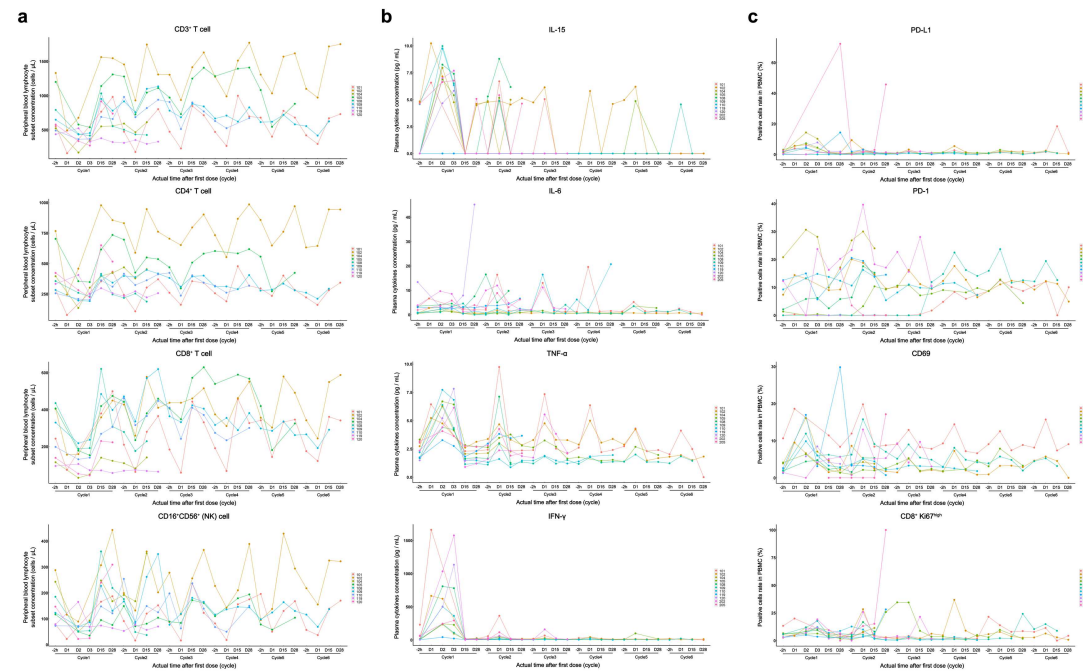
injections of entecavir or VG161. The IL-15R α expression levels were comparable between the entecavir and VG161 groups (Extended Data Fig. 2c). *In vitro* assays confirmed that commonly prescribed anti-hepatitis B agents minimally affected VG161 cytotoxicity (Extended Data Fig. 2e).



Extended Data Fig. 2| Exclusion of the possible effect of anti-HBV agents on VG161 replication *in vitro* and *in vivo*. **a, b**, Comparison of the plasma VG161 concentration between patients who received or did not receive oral administration of anti-HBV agents within 24 h. **c**, Female NCG mice were implanted subcutaneously with Hep3B cells and gavaged with entecavir (0.1 mg/kg/day) or NS (normal saline). Intratumoral injection of 1×10^7 PFU VG161 was performed 3 days after gavage. Copies of the viral genome of VG161 in lung, spleen, liver, heart, and kidney tissues; blood; and the tumor mass were quantified by qPCR at 0.5, 1, 2, 3, and 7 days (n = 3). **d**, The *in vitro* cytotoxicity of VG161 with or without anti-HBV agents against Hep3B cells was determined. Cells were infected with VG161 with or without anti-HBV agent treatment, and cell viability was measured using the CCK-8 assay at 65 h post-infection and normalized to the control cell viability. The cell viability values of different MOI or drug concentrations underwent 4-parameter curve fitting. **e**, The effect of anti-HBV agent treatment on VG161 replication ability in Hep3B cells. Hep3B cells infected with VG161 for 24, 48, and 72 h and the culture supernatant were collected, mixed, and lysed, then titrated using a viral plaque assay. **a–c and e**, Data are the mean $\pm$ standard error. **a–c and e**, Data were determined using the unpaired Student *t*-test (**p* < 0.05; ***p* < 0.01; ****p* < 0.001; *****p* < 0.0001; NS, not significant).

Immunological response

The blood of the dose escalation patients had significantly altered lymphocyte subsets, plasma cytokines, and T-cell immune markers. The CD3⁺, CD4⁺, CD8⁺, and CD16⁺CD56⁺ (NK) cell concentrations initially decreased 24 h after each administration, returning to or exceeding baseline levels by day 15 and 28. Plasma IL-6 levels increased approximately 24 h post-administration in some patients, notably patients 101, 108, and 202, before returning to baseline by day 15 and 28. Similarly, TNF- α and IFN- γ levels increased post-administration and returned to baseline thereafter. This immune response pattern was consistent across multiple treatment cycles (Extended Data Fig. 3a, 3b). The proportions of immune-associated markers (PD-L1, PD-1, CD69, CD8⁺Ki67^{high}) initially increased post-administration and normalized by day 15 and 28 (Extended Data Fig. 3c).



Extended Data Fig. 3| Pharmacokinetic and pharmacodynamic profiles in dose escalation phase.
a, Lymphocyte subset trends in peripheral blood (n = 9). **b**, Plasma cytokine changes in peripheral blood (n = 11). **c**, Changes of typical T cell markers representing immune state in peripheral blood (n = 11).

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164 **Safety profile**

165 VG161 demonstrated a favorable safety profile throughout the study with no DLTs. The
166 MTD was not reached during the dose escalation phase. Extended Data table 1
167 comprehensively summarizes the treatment-related AEs (TRAEs). The most common
168 TRAEs included pyrexia (86.4% of patients), hypoalbuminemia and anemia (each,
169 65.9%) and decreased lymphocyte counts (84.1%), platelet counts (52.3%), neutrophil
170 counts (52.3%), and white blood cell counts (70.5%). Decreased lymphocyte count was
171 the most frequent grade 3 TRAE. Notably, one patient experienced a serious TRAE,
172 (facial paralysis), which resolved following appropriate medical intervention. No grade
173 4 TRAEs or treatment-related deaths were reported.

Extended Data Table 1|Treatment-related adverse events (Dose-group based)

	Dose escalation (PFU)					Dose expansion (PFU)	Total
	1.0×10 ⁸ (n=1)	2.0×10 ⁸ (n=1)	3.0×10 ⁸ (n=3)	4.0×10 ⁸ (n=3)	5.0×10 ⁸ (n=3)	3.0×10 ⁸ (n=33)	(n=44)
Adverse event-Term	n (%)	n (%)	n (%)	n (%)	n (%)	n (%)	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%)
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%)

Extended Data Table 1|Treatment-related adverse events (Dose-group based)

Adverse event-Term	Dose escalation (PFU)					Dose expansion (PFU)	Total
	1.0×10 ⁸ (n=1)	2.0×10 ⁸ (n=1)	3.0×10 ⁸ (n=3)	4.0×10 ⁸ (n=3)	5.0×10 ⁸ (n=3)	3.0×10 ⁸ (n=33)	(n=44)
	n (%)	n (%)	n (%)	n (%)	n (%)	n (%)	n (%)
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%)
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%)

Efficacy in patients with HCC

Due to the small number of ICC patients ($n = 4$), we focused on the 40 HCC patients. Three patients withdrew informed consent form (ICF) before the initial tumor assessment, leaving 37 for evaluation. According to the modified Response Evaluation Criteria in Solid Tumors (mRECIST), 7 patients achieved a partial response (PR), and 17 maintained stable disease (SD). The ORR was 18.92% (95% confidence interval [CI], 7.96-35.16), and the DCR was 64.86% (95% CI, 47.46-79.79). Fig. 1 depicts the changes in the target lesion size from baseline and the treatment durations. To objectively assess VG161's efficacy, we excluded 2 patients who only failed first-line treatment and 1 patient who died of COVID-19. Among the remaining 34 patients, 6 achieved a PR and 16 maintained SD. The ORR was 17.65% (95% CI, 6.76-34.53), and the DCR was 64.71% (95% CI, 46.49-80.25).

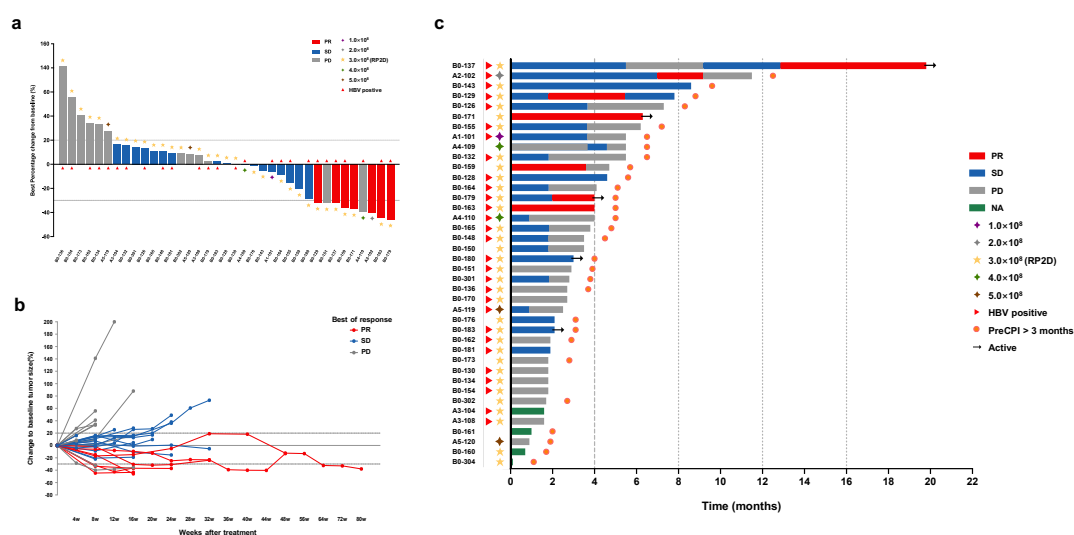


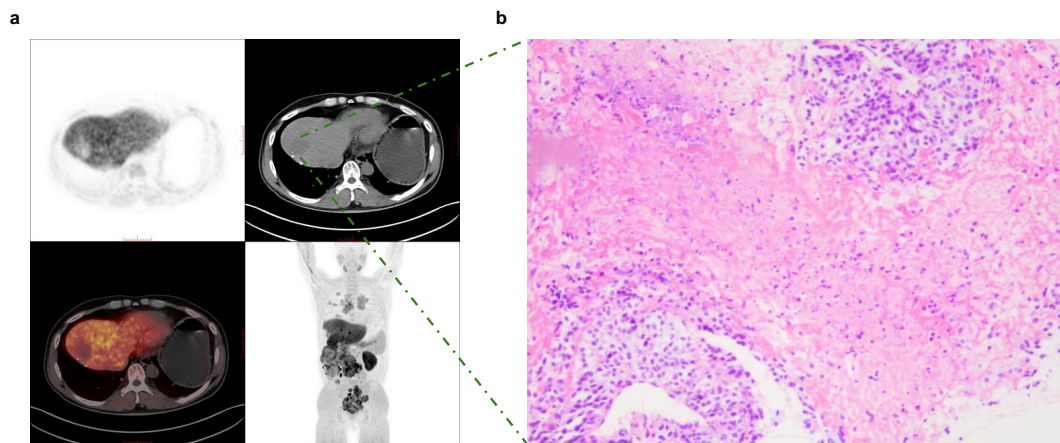
Fig. 1| Treatment response based on mRECIST. a, Waterfall plot depicting the best percentage change in target lesions from baseline ($n = 37$). **b,** Chronological change of target lesions from baseline ($n = 37$). **c,** Duration of treatment from baseline ($n = 40$).

Therapeutic response to VG161 in HCC

The VG161-treated patients with HCC demonstrated significant therapeutic responses characterized predominantly by necrosis in the treated lesions. For example, one patient exhibited no noticeable enhancement in the targeted lesion on CT after one treatment cycle (Fig. 2a). 18F-fluoromethylcholine (18F-FCH) positron emission tomography (PET)/CT analysis revealed significantly reduced tracer uptake, confirming extensive necrosis (Extended Data Fig. 4a). Pathological assessments post-biopsy verified substantial necrosis in the lesions (Extended Data Fig. 4b). A recurring observation was the initial enlargement of lesions, followed by substantial necrosis. The CT scans revealed that the injected and non-injected lesion size increased initially, but necrosis became evident at the core as treatment continued, culminating in near-complete necrosis (Fig. 2b). Another patient had increased lesion size and activity on enhanced CT and ultrasound after two treatment cycles. However, extensive necrosis was observed after four cycles (Fig 2c). Interestingly, non-injected lesions often responded more favorably than injected lesions, demonstrating more significant size reductions and necrosis (Fig. 2a, 2b, 2d, 2e). One patient who presented with subcutaneous and deep abdominal tumors, the latter deemed inoperable due to invasion into major blood vessels, had dramatic post-treatment tumor shrinkage, facilitating successful surgical resection (Fig. 2e).

Why the non-injected lesions responded better to VG161 than the injected lesions were investigated using scRNA-seq and spatial transcriptome analyses of surgical specimens from one patient with HCC (Fig. 2e). The analyses identified 8,035 spots

with a median depth of 1,607 genes per spot and 5,774 unique molecular identifiers (UMIs) from the injected and non-injected lesions (Fig. 2e). Integrating spatial transcriptome data with the scRNA-seq results using Seurat label transfer revealed that the injected lesions were enriched with epithelial cells, whereas the non-injected lesions had higher T/NK cell and fibroblast infiltration (Fig. 2g, 2h). stLearn spatial ligand–receptor analysis highlighted enhanced cellular interactions in the non-injected lesions, particularly among T/NK cells, suggesting more robust immune activation than the injected lesions (Fig. 2i, 2j).



Extended Data Fig. 4| 18F-FCH PET/CT and pathological examination of patient no. A4-109 from Fig. 2. a, The patient’s 18F-FCH uptake was significantly reduced, indicating tumor necrosis. **b,** A puncture biopsy was performed at the injection site after the VG161 treatment, and the pathological results demonstrated typical tumor cell necrosis.

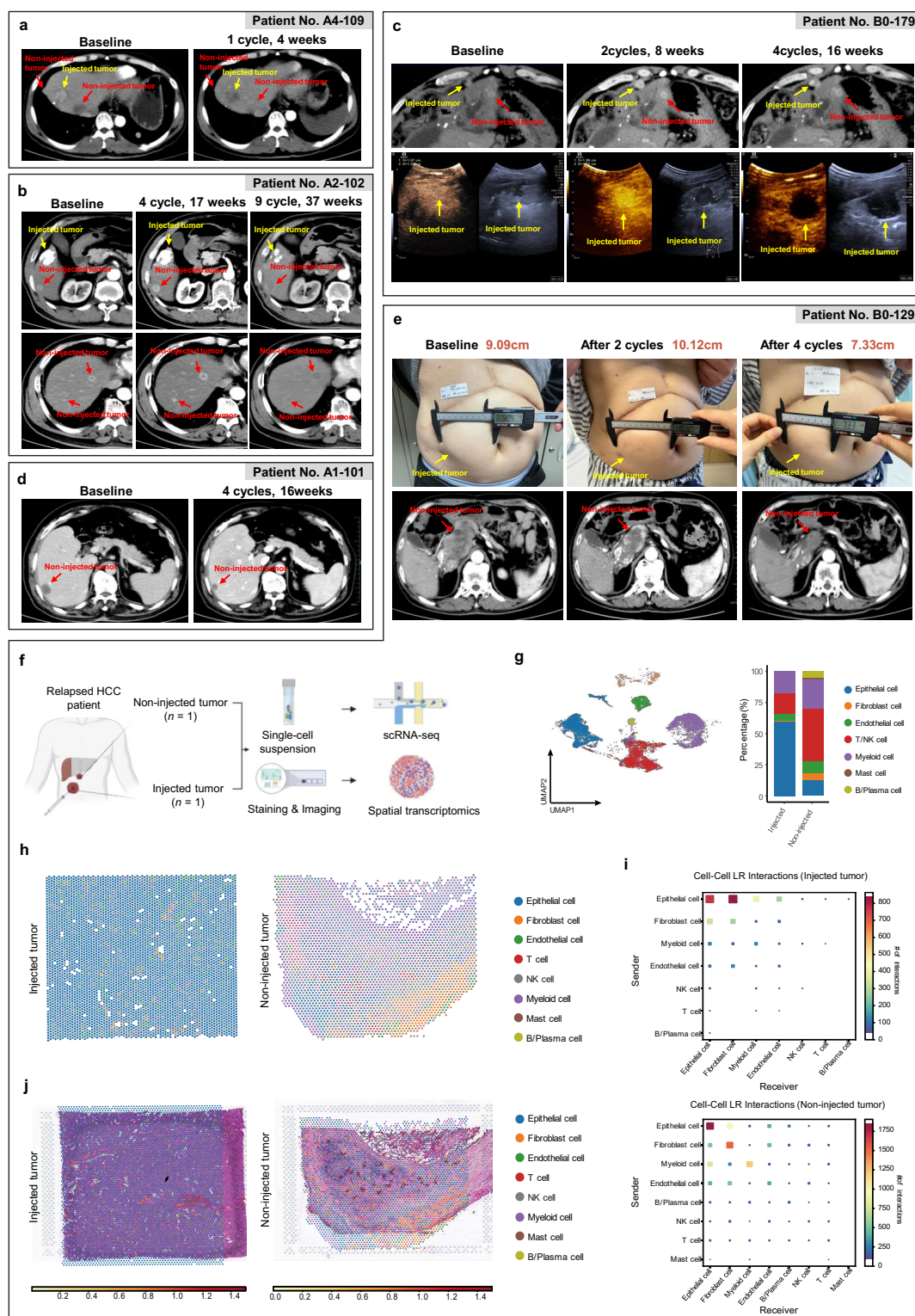


Fig. 2| Representative tumor response data of patients who responded. **a**, Patient no. A4-109 had significantly necrotic injected lesions, and the non-injected lesions were significantly reduced on days 1, 2, and 3 after only one cycle of 1.7×10^8 PFU VG161 (dose level 4). **b**, Patient no. A2-102 received 1.0×10^8 PFU VG161 on days 1 and 2 (dose level 2) and demonstrated a necrotic and shrunken injection site, while necrosis followed the initially enlarged non-injection site. **c**, The injected and non-injected sites of patient no. B0-179, who received 1.0×10^8 PFU VG161 on days 1, 2, and 3 (dose level 3),

demonstrated initially enlarged but subsequently necrotic neoplasms. **d**, The non-injected lesions of patient no. A1-101 shrank after receiving 1.0×10^8 PFU VG161 on day 1 (dose level 1) after four cycles. **e**, After patient no. B0-129 received dose level 3 VG161 for four cycles, the non-injected lesions significantly shrank in necrosis and the patient underwent radical surgery in December 2022. **f**, Surgical specimens dissected from patient no. B0-129 underwent scRNA-seq and spatial transcriptome RNA-seq analyses. **g**, UMAP plot depicting clusters of all cell types from one patient analyzed by scRNA-seq (left). Bar plot illustrating the proportion of main cell types in the patient (right). **h**, Spatial scatter plot depicting the proportions and distribution of cell types. **i**, Heatmap illustrating the spatial interaction strength between interacting cells. **j**, Spatial scatter plot visualizing the mean ligand–receptor expression according to the color gradient.

Survival analysis

Survival analysis was conducted on 37 HCC patients with evaluable tumors. The median PFS was 2.9 months (95% CI, 1.81-3.70), and the OS was 12.4 months (95% CI, 7.10-20.10), as shown in Fig. 3a, 3b. After excluding 2 patients who only failed the first-line treatment and 1 patient who died of COVID-19, the median PFS and OS were 2.8 months (95%CI: 1.85-3.95) and 9.40 months (95%CI: 0.47-18.33), respectively (Fig. 3c, 3d). The 6- and 12-month survival rates were 80.2% and 47.8%, respectively (Extended Data Table 2). A subgroup of 22 patients who had been on CPI therapy for > 3 months before enrolment (PreCPI > 3 m) had notably extended PFS and OS compared to PreCPI $\leq$ 3 m patients. The median PFS in this subgroup was 3.6 months versus 1.8 months ($p = 0.04$; Fig. 3e), and the median OS was significantly longer (17.30 months vs. 7.40 months; $p = 0.04$) (Fig. 3f). Patients who underwent post-systemic therapy (PST) following VG161 treatment also had enhanced survival (median OS: 20.10 months vs. 8.80 months for those who received no further anti-cancer treatment; $p = 0.032$) (Fig. 3h). Assessment of the effects of oral anti-HBV on survival outcomes revealed no significant differences in the median PFS or OS between patients who used

and did not use these agents (Fig. 3i, 3j). Extended Data Table 3 details the baseline characteristics of these subgroups and their effects on survival. PreCPI > 3 m and PST following VG161 treatment were significant independent variables affecting OS ($p = 0.02$).

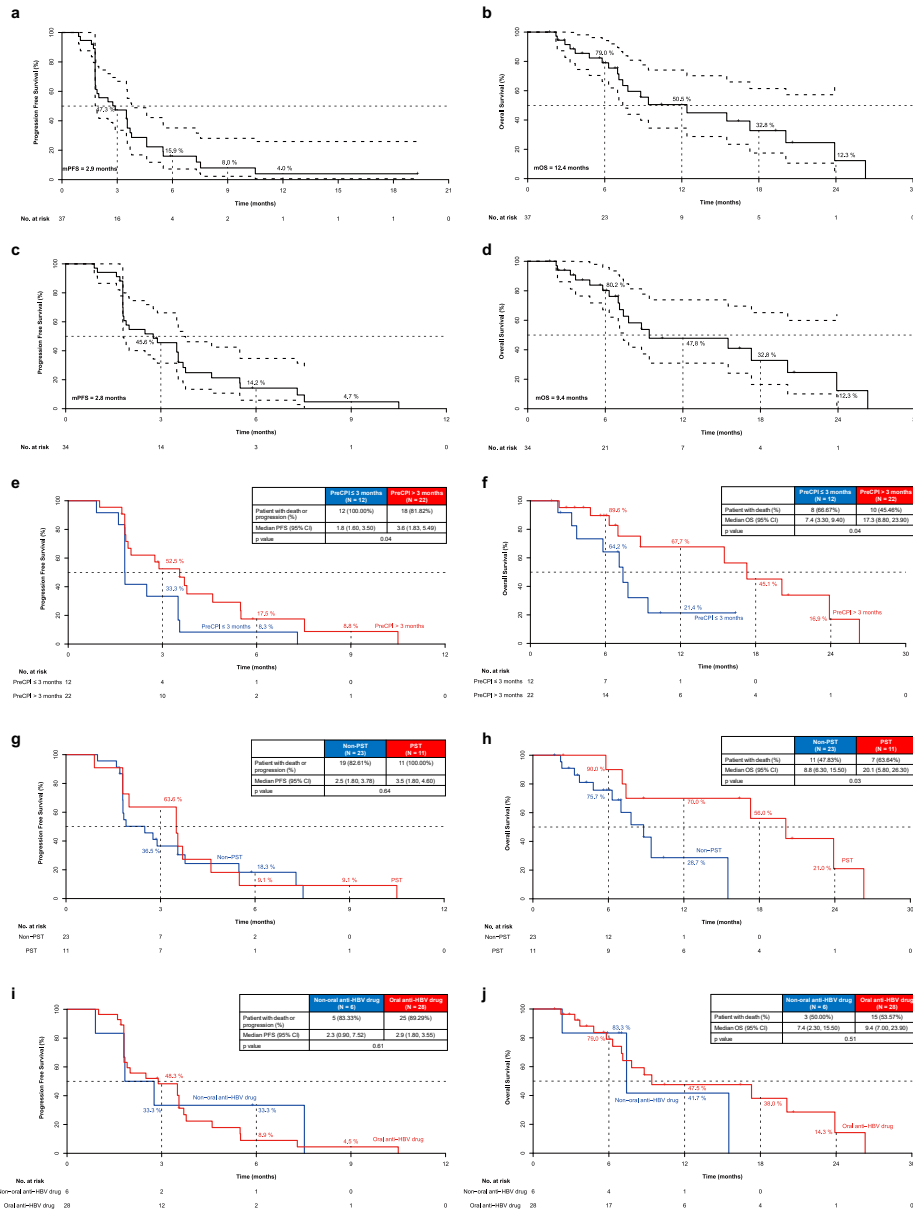


Fig. 3| KM curves from baseline. a, PFS from baseline in patients with HCC. **b**, OS from baseline in patients with HCC. **c**, PFS from baseline in patients who failed second-line therapy. **d**, OS from baseline in patients who failed second-line therapy. **e**, PFS based on preCPI status from baseline. **f**, OS based on preCPI status from baseline. **g**, PFS based on PST status from baseline. **h**, OS based on PST status from baseline. **i**, PFS based on anti-HBV status from baseline. **j**, OS based on anti-HBV status from baseline. Subgroups were compared using the log-rank tes

Extended Data Table 2 Survival comparison between VG161-C102 and historical control					
Trial or registration number (phase)	Population	Arm	Median OS	6m OS (%)	12m OS (%)
RESORCE ¹ (III)	Sorafenib failed	Regorafenib	10.8	70	45
		Placebo	7.8	59	29
	KEYNOTE-240 ² (III)	Sorafenib failed or intolerance	Pembrolizumab	13.9	77
Saline placebo			10.6	73	48
NCT01908426 ³ (III)	Sorafenib failed only	Cabozantinib	11.3	75	49
		Placebo	7.2	55	33
AHELP ⁴ (III)	≥1L chemo/target failed or intolerance	Apartinib	8.7	70	37
		Placebo	6.8	56	29
NCT02989922 ⁵ (II/III)	1L failed in China	Camrelizumab	14.0	76	56
VG161-C102	entire cohort		12.4	79	50.5
VG161-C102 failed after 2L group	34 failed after 2L	VG-161	9.4	80.2	47.8
VG161-C102 PreCPI>3 m group	22 failed after 2L		17.3	89.6	67.7
VG161-C102 PST group	11 failed after 2L		20.1	90	70
Abbreviation: 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).					

Extended Data Table 3 Demographic and baseline characteristics of subgroups						
	PreCPI ≤ 3 months n=12 (%)	PreCPI > 3 months n=22 (%)	<i>P</i> value	PST n=11 (%)	non-PST n=23 (%)	<i>P</i> 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						

Extended Data Table 3| Demographic and baseline characteristics of subgroups

	PreCPI ≤ 3 months n=12 (%)	PreCPI > 3 months n=22 (%)	<i>P</i> value	PST n=11 (%)	non-PST n=23 (%)	<i>P</i> 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

Tumor microenvironment (TME) dynamics under VG161 treatment

The effects of VG161 on the HCC TME were examined pre- and post-treatment using scRNA-seq, scTCR-seq, and scBCR-seq of biopsy samples from four patients with HCC (Fig. 4a). A total of 116,677 cells that passed rigorous quality control measures were analyzed. Data were integrated using the Harmony algorithm to correct for batch effects. Cell types were characterized using Uniform Manifold Approximation and Projection (UMAP) and canonical marker annotation. This analysis identified 33 distinct cell clusters: four epithelial, four fibroblast, one endothelial, nine T/NK, 12 myeloid, one mast, and two B/plasma cell clusters (Fig. 4b, 4c).

The post-treatment samples demonstrated a notable shift in cellular composition: epithelial and fibroblast cells were decreased, while T/NK and myeloid cells were increased, indicating dynamic TME remodeling (Fig. 4d, 4e). Enhanced proportions of T/NK cells (CD8-GZMK, CD8-GZMH, CD8-LAG3, CD4-CCR7, CD4-CTLA4, CD4-FOXP3, CD8/4-STMN1, NK-GNLY) were observed. Myeloid cells (Macro-ISG15, cDC2-CD1C, cDC3-LAMP3, Myeloid-STMN1) were also increased post-treatment (Fig. 4f). TCR analysis revealed significant clonal expansion post-treatment, particularly in the CD4-CCR7 and CD8-GZMK subsets, suggesting active T-cell responses (Fig. 5a, 5b). The scBCR-seq revealed minimal differences in clonal expansion between pre- and post-treatment (Fig. 5a, 5b).

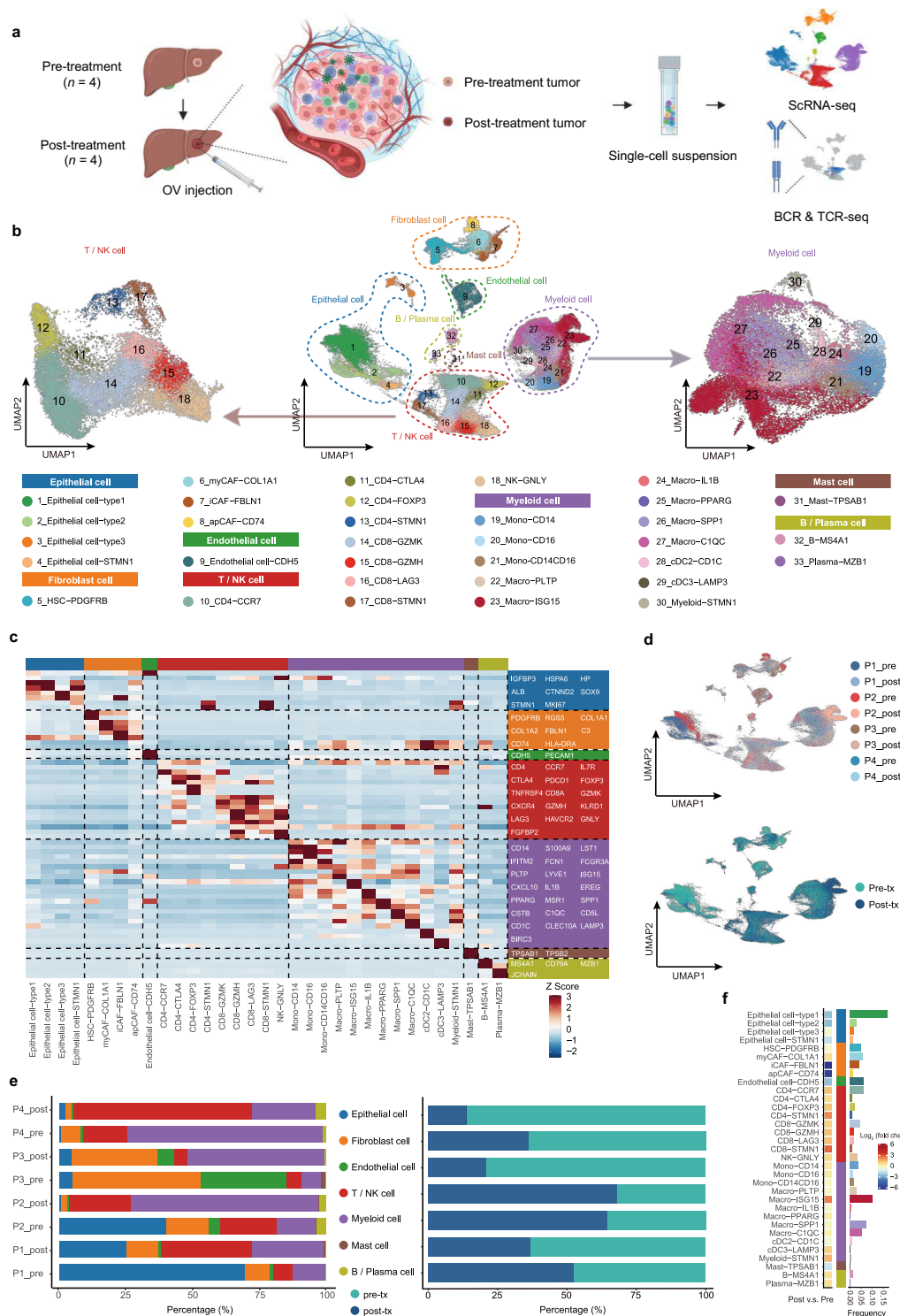


Fig. 4| Changes in TME immune landscape following VG161 treatment. a, Schematic graph presenting the overall experimental strategy. **b**, UMAP plot depicting the clusters of all cell types from four patients with HCC analyzed using scRNA-seq. **c**, Heatmap illustrating the expression of typical marker genes in the specific cell type. **d**, UMAP plot depicting the cell origins by color in patients and groups. **e**, Bar plot showing the proportion of seven main cell types in patients and groups. **f**, Heatmap depicting the ratio of all 33 cell types in post- and pre-treatment groups. P1: Patient no. B0-181; P2: patient no. B0-179; P3: patient no. B0-183; P4: patient no. B0-180.

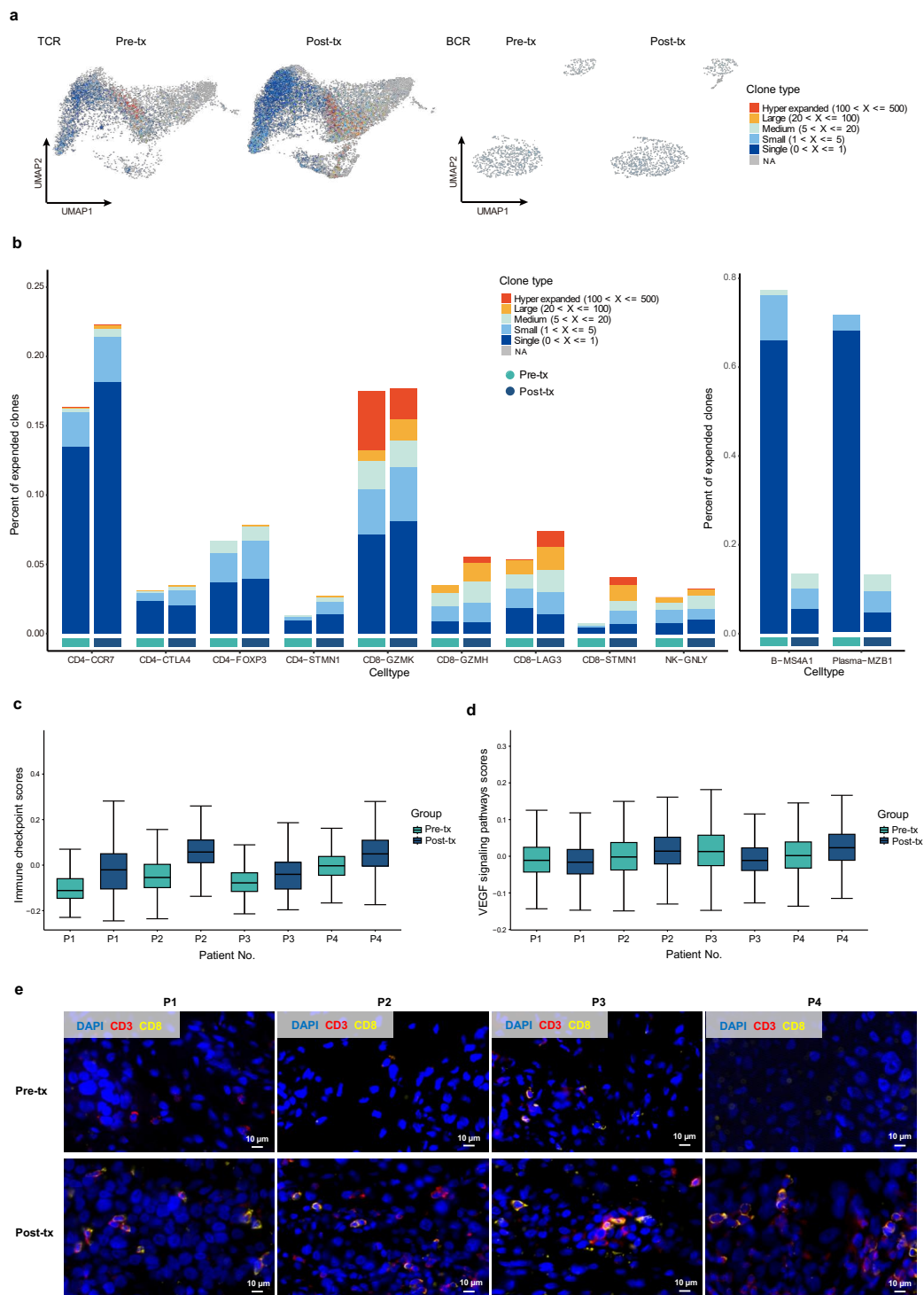


Fig. 5| VG161 treatment modulates the TME and enhances T cell infiltration in tumors. a, UMAP plot depicting the T/NK and B/plasma cells according to the clonal expansion status. **b,** Bar plot depicting the expanded clonotypes in T/NK and B/plasma cells. **c,** Immune checkpoint score changes pre- and post-treatment. **d,** VEGF signaling pathway score changes pre- and post-treatment. **e,** T cells in biopsies from a 4-patient cohort were evaluated using mIF staining before (Pre-tx) and after (Post-tx) OV therapy. P1: Patient no. B0-181; P2: patient no. B0-179; P3: patient no. B0-183; P4: patient no. B0-180.

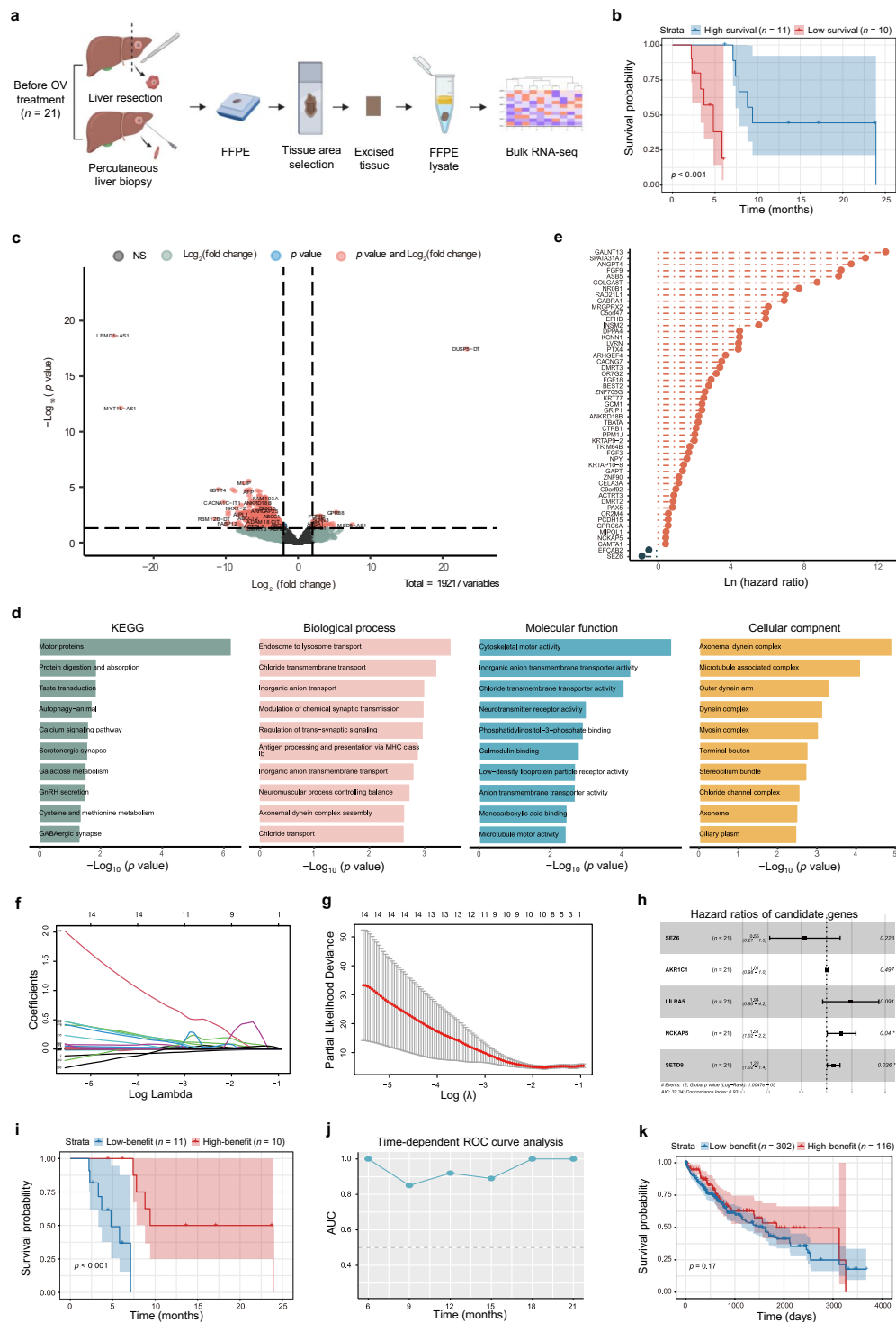
The post-treatment samples demonstrated increased immune checkpoint gene signatures (Fig. 5c), indicating enhanced tumor sensitivity to CPIs. The mIF confirmed increased CD3⁺ and CD8⁺ T cell infiltration post-treatment, supporting the VG161-facilitated immunogenic shift in the TME (Fig. 5e).

Genomic insights and prediction modeling in HCC

Baseline tumor samples from 27 patients were analyzed, excluding four patients with ICC, one patient who failed first-line treatment, and one patient who died from COVID-19, leaving 21 HCC samples for RNA-seq analysis (Extended Data Fig. 5a). Differentially expressed genes (DEGs) were identified (49 upregulated and 745 downregulated) and categorized by survival outcomes according to median OS ($p < 0.001$; Extended Data Fig. 5b). Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analyses revealed significant enrichment in the motor protein, protein digestion, absorption, and calcium signaling pathways. GO analysis highlighted significant activity in endosome to lysosome transport, cytoskeletal motor activity, and axonemal dynein complex (Extended Data Fig. 5d). Univariate Cox regression analysis identified 299 potential prognostic genes from among the DEGs (Extended Data Fig. 5e).

An efficacy prediction model was constructed using a LASSO Cox regression model and incorporated five risk genes: *SEZ6*, *AKR1C1*, *LILRA5*, *NCKAP5*, and *SETD9* (Extended Data Fig. 5f, 5g). The model was validated using a risk score derived from multivariate Cox regression analysis, identifying *NCKAP5* and *SETD9* as

significant independent predictors ($p < 0.001$; Extended Data Fig. 5h). The model predictive accuracy was assessed using KM curves and time-dependent receiver operating characteristic (ROC) curves. The patients were categorized into high- and low-benefit groups using the median risk score, with the KM curve demonstrating a significantly higher survival rate for the high-benefit group (Extended Data Fig. 5i). Similarly, the time-dependent ROC curve demonstrated that the model accurately predicted the 6-, 9-, 12-, 15-, 18-, and 21-month survival rates ($p = 0.17$; Extended Data Fig. 5j). The model was also tested against survival data from 418 patients with advanced HCC from The Cancer Genome Atlas (TCGA-LIHC) who had not received such treatments. The analysis indicated no significant differences in survival outcomes between the model-defined high- and low-benefit groups (Extended Data Fig. 5k). Clinical integration was facilitated using an initial version of the ViroPredict 1.0 model (<https://viropredict.github.io/>).



Extended Data Fig. 5| Efficacy prediction model for VG161 in advanced HCC. a, Graphic overview of the experimental strategy. **b**, KM survival curves between low- and high-benefit groups. **c**, Volcano plot for DEGs of low- and high-benefit groups. **d**, GO and KEGG analysis of DEGs. **e**, Univariate Cox regression analysis identification of the prognosis-associated DEGs. **f**, **g**, LASSO Cox regression analysis assessment of the prognostic and screen risk genes. **h**, Multivariate Cox regression analysis of risk genes. **i**, KM survival curves based on the risk model. **j**, ROC curves based on the risk model. **k**, KM survival curves based on the risk model in the TCGA-LIHC cohort.

351

352 **Discussion**

353 This is the first evaluation of VG161 safety and efficacy in a clinical trial, where it is a
354 novel OV targeting HCC post-JX-594. VG161 prioritizes safety as it is engineered from
355 an HSV backbone without the neurotoxic gene ICP3.45. Pyrexia was the most common
356 TRAE, occurring in 86.4% of patients, comparable to JX-594 and G47 Δ outcomes^{14,21}.
357 Typically, pyrexia manifested within 6 h post-administration, but patients typically
358 recovered by the following day with immediate antipyretic and supportive care. As viral
359 replication peaked 24-48 h post-injection (before the release of immune-stimulating
360 factors by VG161 and considering that all patients have HSV antibodies), the pyrexia
361 likely stemmed from an antiviral response. Interestingly, pyrexia tended to intensify
362 during the second treatment cycle, possibly due to the activation of pre-existing
363 antiviral antibodies, maintaining an alert antiviral immune state. However, recovery to
364 normal body temperature by the next day was consistently observed. AEs such as
365 cytopenia were primarily characterized by decreased lymphocyte and platelet counts
366 post-administration. While low platelet count presented a bleeding risk due to the
367 intratumoral injection method, it was transient and fully reversible post-treatment
368 cessation. We maintained safety thresholds rigorously to mitigate the
369 thrombocytopenia-associated bleeding risks.

370 The mRECIST proposed in 2010²² and revised in 2020²³ introduced the concept of
371 “active” tumors to measure intrahepatic liver cancer lesions more accurately, addressing
372 the issue of non-shrinking lesions in traditional assessments. HSV-1-based OVs

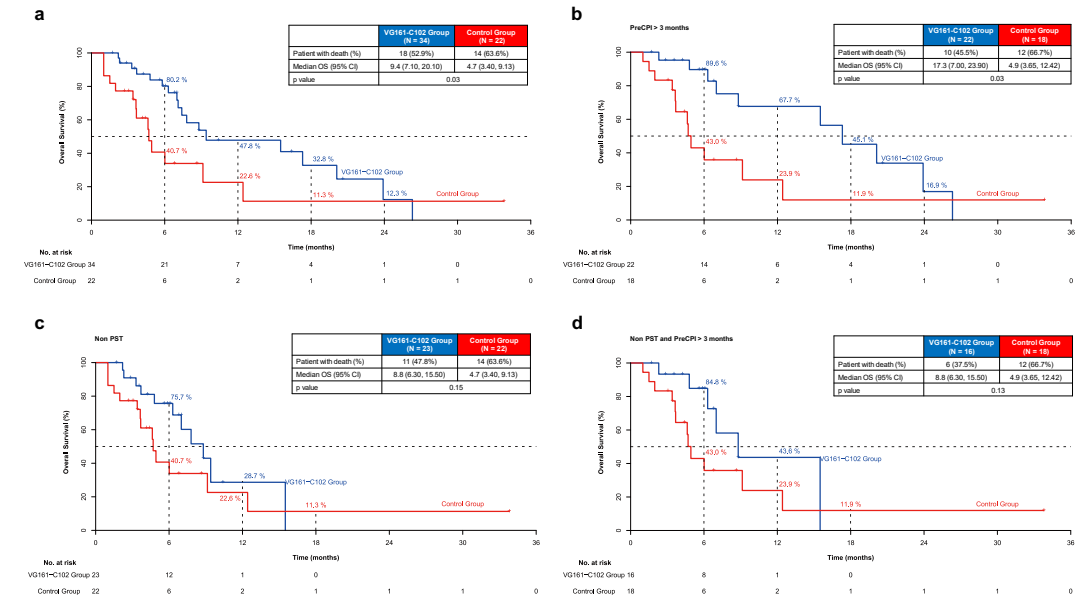
disrupted neoplasms by targeting tumor angiogenesis^{24,25}. We used contrast-enhanced CT, magnetic resonance imaging (MRI), PET-CT, and needle biopsies with patient consent, all confirming the presence of tumor necrosis. Necrosis was predominantly observed under VG161 administration, prompting the adoption of mRECIST as the primary tumor evaluation criterion to ensure a more objective assessment of efficacy. Compared to the phase III RESORCE trial (NCT01774344)⁶, which also used mRECIST for evaluation, the VG161 ORR was higher (17.65% vs. 10%). Importantly, while the RESORCE trial included patients who had only failed first-line treatment (specifically sorafenib), all 34 patients in our study had failed at least second-line treatments. These results underscored the potential of VG161 for treating advanced-stage HCC and should be evaluated in further clinical trials.

OS is considered the gold standard for assessing patient benefit in oncology trials. In the present study, the OS of the patients with HCC was 9.4 months, aligning with results from several large-scale phase II/III clinical trials^{5,6,26-29} despite our cohort being treated in later lines of therapy. Extended Data Table 4 details the survival data. Subgroup analysis of the survival benefits among the patient groups revealed that PreCPI > 3 m patients exhibited improved survival outcomes. This observation supported the hypothesis that prolonged CPI exposure enhances immunotherapy responsiveness, suggesting that PreCPI > 3 m could effectively predict VG161 efficacy. The baseline demographic and clinical characteristics between the subgroups (Extended Data Table 3) were not significantly different, indicating that VG161 treatment and not pre-existing patient characteristics theoretically accounted for the observed survival differences.

These results underscored the potential of VG161 to enhance survival in later-line treatment settings. The results should be confirmed in larger, more diverse patient populations.

As our dataset consisted exclusively of historical controls and did not align with the treatment lines, we attempted to demonstrate the efficacy of VG161 more accurately. We meticulously compiled data from patients with HCC at our center who had received only the best supportive care (BSC) following the failure of systemic treatments in recent years and established them as a control group. The inclusion criteria for these patients were: (1) received at least two lines of systemic therapy with documented progression; (2) no prior treatment with VG161 or other OV therapies; and (3) compliance with the main study entry criteria. All controls provided written informed consent for the use of their data in this study. Ultimately, the control group comprised 22 patients with HCC. The analysis revealed that the VG161-treated patients had significantly longer OS than the controls (median OS: 9.40 months vs. 4.70 months; $p = 0.03$; Extended Data Fig. 6a). Subgroup analysis revealed that VG161-treated PreCPI > 3 m patients had a markedly better median OS than the controls (17.30 months vs. 4.93 months; $p = 0.03$; Extended Data Fig. 6b). Comparison of the survival of non-PST patients with that of the controls revealed that the median OS was 8.80 months versus 4.70 months ($p = 0.15$; Extended Data Fig. 6c). The PreCPI > 3 m patients in the non-PST group also had significantly improved survival compared to the controls (median OS: 8.80 months vs. 4.93 months; $p = 0.13$; Extended Data Fig. 6d). While the current level of evidence is not definitive, it nonetheless provides substantial confidence and

motivation to continue our investigations and validate these results.



Extended Data Fig. 6 | The contrast of KM curves between the VG161-C102 group vs. control group.

a, Overall OS in the VG161-C102 group vs. control group. **b**, Comparison of the OS between the VG161-C102 and control groups in the >3 m subgroup. **c**, OS of the non-PST and control groups in the VG161-C102 group. **d**, Comparison of the OS between the non-PST+PreCPI > 3 m patients in the VG161-C102 group and the PreCPI > 3 m patients in the control group. Subgroups were compared using the log-rank test.

Transcriptomic analyses of patient samples pre- and post-VG161 treatment revealed substantially increased cytotoxic T cells within the TME. Notably, TCR repertoire analysis of T-cell clonality in the VG161-treated tumors indicated pronounced expansion. Although all patients tested HSV-1-positive before VG161 treatment, we cannot rule out that this expansion included an increase in anti-HSV T cells. However, the increased T-cell clones might also have derived from the spread of tumor antigens following OV-induced tumor cell lysis. We hypothesized that VG161 might initiate a potent intratumoral vaccination effect, enhancing anti-tumor immunity. This hypothesis was supported by the abscopal effect in certain patients, where robust immune

responses were noted in non-injected tumors, paralleled by significant regression. These observations underscored the potential of VG161 as a catalyst for systemic anti-tumor immune responses, emphasizing its therapeutic promise in oncology.

Notably, the patients who received PST post-VG161 had significant survival benefits. These patients were previously resistant to systemic therapies and experienced tumor progression, and VG161 appeared to reverse their resistance. scRNA-seq and mIF analysis of the biopsy samples from four patients with HCC indicated substantially increased T, NK, and myeloid cells within the TME post-treatment. This result suggested that VG161 stimulated anti-tumor immunity and transformed the HCC immune landscape from “cold”, which is typically resistant to systemic (especially immunotherapeutic) treatments, to “hot”. Interestingly, this transformation was more pronounced in the non-injected lesions, possibly due to the localized immune modulation at the injection sites by the viral infection, which may involve immune suppression upregulation^{30,31} and antiviral immune response competition. We previously confirmed that the virus could not infect non-injected lesions through the blood; therefore, the changes in TME are directly caused by the anti-tumor immune responses elicited by VG161²⁰. Furthermore, our results demonstrated increased immune checkpoint gene signature scores post-VG161 in the four patients, supporting the continued use of CPIs as a follow-up treatment.

VG161 is a promising pivotal component of sandwich therapy (systemic therapy-OV therapy-systemic therapy) in HCC treatment strategies. Additionally, preclinical studies demonstrated that VG161 combined with the atezolizumab, and bevacizumab regimen

influence VG161 therapeutic efficacy is critical. Our data indicated that the commonly used anti-HBV agents did not affect virus replication or the prognosis of VG161-treated patients. This was likely due to the structural differences between HBV and HSV³⁴ rendering anti-HBV agents ineffective against HSV.

Identifying reliable efficacy markers is crucial in cancer treatment as it helps identify the patients most likely to benefit from specific treatments and aids in optimizing medical resource allocation. Despite the limited scope of our sample size, this study introduced an innovative efficacy evaluation model, ViroPredict 1.0, designed specifically for assessing OV therapy in HCC. This model leverages genetic data from five key genes (*SEZ6*, *AKR1C1*, *LILRA5*, *NCKAP5*, *SETD9*) identified as critical indicators of therapeutic response. The preliminary results confirmed that ViroPredict 1.0 is statistically robust and biologically relevant, providing valuable insights into the potential therapeutic outcomes. We will refine and enhance ViroPredict 1.0 as we expand our research in the ongoing clinical trial (NCT06124001) and subsequent studies. We aim to increase the accuracy and predictive power of the model by incorporating a larger dataset, facilitating more personalized and effective treatment strategies for patients with HCC.

The primary study limitations are the small sample size and single-arm design. Our main goal was to evaluate VG161 safety and preliminary efficacy. We conserved clinical resources by intentionally limiting the cohort size to achieve the necessary statistical power. However, the promising results encourage the expansion of our research. We intend to validate and extend our findings by conducting further

multicenter pivotal trials that will include a more diverse patient population, particularly extending beyond the Asian demographic. Additionally, the development of the ViroPredict 1.0 model was limited by the small sample number, rendering widespread application premature. We aim to refine and enhance the model accuracy and reliability as we obtain more data from upcoming clinical trials.

Conclusion

VG161 had a favorable safety profile in treating liver cancer, demonstrating significant therapeutic potential. This study focused on patients with HCC who were refractory after receiving at least two prior lines of therapy (third-line patients). VG161 reshaped the tumor immune microenvironment and demonstrated comparable efficacy to second-line therapies. Furthermore, VG161 significantly extended survival in a subgroup of patients in line with predictions from prior treatment responses or our novel efficacy prediction model. This result underscored the urgent need for additional research to expand the patient cohort who might benefit from this treatment. Integrating systemic therapy before and after VG161 administration also highlighted the potential of combination strategies to enhance clinical outcomes. Moreover, our study provides a groundbreaking insight, confirmed through animal and human studies, that anti-hepatitis B agents do not compromise OV replication and efficacy. This discovery opens new avenues for safe VG161 application in patients with concurrent hepatitis B, enhancing its clinical utility and relevance.

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Methods

Study design and participants

This phase I, open-label, single-arm study evaluated VG161 safety, pharmacokinetics, and anti-tumor activity in patients with advanced primary liver cancer. The dose escalation phase involved an accelerated titration plus 3+3 design at 1.0×10^8 plaque-forming units (PFU) on day 1 (dose level 1); 1.0×10^8 PFU on days 1 and 2 (dose level 2); 1.0×10^8 , 1.3×10^8 , or 1.7×10^8 PFU on days 1, 2, and 3 (dose level 3-5), respectively, of each 28-day cycle. Dose escalation continued until ≥ 2 dose-limiting toxicities (DLTs) were observed in a dose level in 3-6 patients. When ≥ 2 DLTs occurred in the first 28 days of a dose level, the next lower dose level was deemed the maximum tolerated dose (MTD). Based on the results, 1.0×10^8 PFU on days 1, 2, and 3 (dose level 3) was recommended for the dose expansion phase involving a single-arm cohort and Simon's minimal maximum two-stage design. The study was registered with Clinical Trial.gov (NCT04806464).

The patient inclusion criteria were: 1) 18-75 years old; 2) had advanced primary HCC, intrahepatic cholangiocarcinoma (ICC), or mixed liver cancer confirmed by histology or cytology and had failed standard treatment with limited therapeutic option; 3) availability of liver tumor lesions and/or extrahepatic metastases that could be injected under ultrasound or computed tomography (CT) guidance with an injection lesion diameter > 1.5 cm (lymph node lesion has a short diameter); 4) Eastern Cooperative Oncology Group (ECOG) score 0-1; 5) expected survival time > 3 months; 6) full organ function.

The First Affiliated Hospital of Zhejiang University School of Medicine, Hunan Cancer Hospital, and Zhongshan Hospital Institutional Review Boards (IRB) approved the study protocol.

Endpoints

The primary endpoint of the dose escalation phase was VG161 safety and tolerability, assessed by monitoring the AEs, serious AEs, clinical laboratory measurements, and physical examinations. AEs were categorized according to severity (National Cancer Institute Common Terminology Criteria for Adverse Events v5.0) and the relationship with the investigated product. The secondary endpoints were efficacy and pharmacokinetics/pharmacodynamics. Tumor response was evaluated using the immune Response Evaluation Criteria in Solid Tumors (iRECIST), RECIST 1.1, and modified RECIST (mRECIST) and included the objective response rate (ORR), disease control rate (DCR), progression-free survival (PFS), and overall survival (OS). The pharmacokinetic parameters included the VG161 DNA concentration pre- and post-VG161 administration. The pharmacodynamic parameters included peripheral blood lymphocyte subsets ($CD3^+$, $CD4^+$, $CD8^+$, $CD4^+/CD8^+$ ratio, $CD19^+$, $CD16^+CD56^+$ (natural killer [NK]) cells), plasma cytokines (IL-15, IL-6), T cell immunity, and other peripheral blood immune-related indicators (PD-L1, PD-1, CD69, $CD8^+Ki67^{high}$). Viral shedding was assessed by detecting VG161 DNA in blood, urine, feces, oral mucosa, and the injection site using quantitative PCR (qPCR). The primary endpoint of the dose expansion phase was the ORR. The secondary endpoints were efficacy indicators (ORR,

DCR, PFS, OS), safety, and pharmacodynamics parameters.

VG161 preparation and intratumoral administration

A clinical-grade VG161 product was prepared at Gensail and OBiO (Shanghai, China) according to Good Manufacturing Practice and stored at -60°C . A tube of clinical lot VG161 was thawed before each administration.

Under ultrasound or CT guidance, the needle was punctured into the active tumor tissue while avoiding blood vessels, and the appropriate volume of VG161 was injected according to the tumor size. The needle was withdrawn during the injection to ensure uniform drug distribution. After the injection, the tumor was rinsed with ~ 0.5 mL normal saline. The needle was removed slowly to avoid leakage at the insertion point. After drug administration, the puncture site was compressed with a cotton swab/sterile gauze, and the injection site was covered with dry sterile gauze and bandaged for fixation.

RNA-sequencing (RNA-seq)

In order to perform RNA-seq, the primary sample was directly obtained from the operating room, and test sample was generated from this primary sample. Specimens were dissociated into single cells according to protocols. Total RNA was extracted using the TRIzol reagent (Invitrogen, CA, USA) according to the manufacturer's protocol. RNA purity and quantification were evaluated using the NanoDrop 2000 spectrophotometer (Thermo Scientific, USA). RNA integrity was assessed using the

Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). Then the libraries were constructed using VAHTS Universal V6 RNA-seq Library Prep Kit (Vazyme, China) according to the manufacturer's instructions. The transcriptome sequencing and analysis were conducted by OE Biotech Co., Ltd. (Shanghai, China).

The libraries were sequenced on a Illumina (San Diego, USA) Novaseq 6000 platform, 150 bp paired-end reads were generated. Raw data (raw reads) of fastq format were firstly processed using the Trimmomatic software³⁵. Sequencing reads were mapped to the human genome (GRCh38) using HISAT2³⁶. For mRNAs, FPKM³⁷ of each gene was calculated using Cufflinks³⁸, and the read counts of each gene were obtained by HTSeq-count³⁹. Differential expression analysis was performed using the DESeq2⁴⁰. Q value < 0.05 and foldchange > 2 or foldchange < 0.5 was set as the threshold for significantly differential expression. For lncRNAs, the transcriptome from each dataset was assembled independently using the Cufflinks 2.0 program³⁸. All transcriptomes were pooled and merged to generate a final transcriptome using Cuffmerge (Cufflinks 2.0). All transcripts that overlapped with known mRNAs, other non-coding RNA and non-lncRNA were discarded. Next, the transcripts longer than 200 bp and the number of exons > 2 were picked out, and the CPC (v 0.9-r2)⁴¹, PLEK (v 1.2)⁴², CNCI (v 1.0)⁴³, Pfam (v 30)⁴⁴ were used to predict transcripts with coding potential. The novel predicted lncRNAs were obtained through these processes. The characteristics (including length, type, number of exons) of lncRNA were analyzed after screening. Then the novel predicted lncRNAs and known lncRNAs (from NCBI and Ensemble database) were both used for expression calculation and differential screening. For circRNAs,

circRNAs were identified using CIRI (v2.0.3)⁴⁵ and the expression of circRNAs were calculated using RPM (spliced reads per million mapping)⁴⁶. Then the differential expression analysis was also using DESeq2. All sequencing process and analyses were performed by OE Biotech Co., Ltd. (Shanghai, China).

Single-cell RNA-seq (scRNA-seq)

In order to perform scRNA-seq, the primary sample was directly obtained from the operating room, and test sample was generated from this primary sample. Specimens were dissociated into single cells according to protocols. After resuspension in 1× phosphate-buffered saline, all single-cell suspensions were loaded into a 10× Chromium Controller (10× Genomics, Pleasanton, CA, USA), aiming for 7000 cells, with the Single Cell 3' v2 reagent kit (10x Genomics), according to the manufacturer's instructions. All libraries were sequenced on Illumina NovaSeq 6000 using a paired-end 150 bp.

The FASTQ files were processed and aligned to GRCh38 human reference genome using Cell Ranger software (version 7.0.1) from 10× Genomics, with unique molecular identifier (UMI) counts summarized for each barcode. The UMI count matrix was then analyzed using Seurat⁴⁷ (version 4.0.0) R package. To remove low-quality cells and likely multiplet captures, a set of criteria were conducted: Cells were filtered by 1) gene numbers (gene numbers < 200); 2) UMI (UMI <1000); 3) log10GenesPerUMI (log10GenesPerUMI < 0.7); 4) percentage of mitochondrial RNA UMIs (proportion of UMIs mapped to mitochondrial genes > 10%) and 5) percentage of hemoglobin RNA

UMIs (proportion of UMIs mapped to hemoglobin genes > 5%). Subsequently, the DoubletFinder⁴⁸ package (version 2.0.3) was used to identify potential doublets. To obtain the normalized gene expression data, library size normalization was processed using the NormalizeData function. Specifically, the global-scaling normalization method “LogNormalize” normalized the gene expression measurements for each cell by the total expression, multiplied by a scaling factor (10,000 by default), and log-transformed the results.

Top 2000 highly variable genes (HVGs) were calculated using the Seurat function FindVariableGenes (mean.function = FastExpMean, dispersion.function = FastLogVMR). Principal-component analysis (PCA) was performed to reduce the dimensionality with RunPCA function. Graph-based clustering was performed to cluster cells according to their gene expression profile with the FindClusters function. Cells were visualized using a 2-dimensional Uniform Manifold Approximation and Projection (UMAP) algorithm with the RunUMAP function. The FindAllMarkers function (test.use = presto) was used to identify marker genes of each cluster. Differentially expressed genes (DEGs) were selected using the function FindMarkers (test.use = presto). P value < 0.05 and |log2foldchange| > 0.58 was set as the threshold for significantly differential expression. GO enrichment and KEGG pathway enrichment analysis of DEGs were respectively performed using R (version 4.0.3) based on the hypergeometric distribution. The sequencing and bioinformatics analysis were provided by OE Biotech Co., Ltd. (Shanghai, China).

Single-cell V(D)J analysis

The cell suspension prepared for single-cell sequencing underwent human T cell receptor and B cell receptor (TCR and BCR, respectively) library analysis. The T and B cell V(D)J was enriched according to a 10× Genomics Chromium™ Single Cell V(D)J Enrichment Kit, Human T Cell (Cat. No.: 1000005, Pleasanton, CA, USA) and Human B Cell (Cat. No.: 1000016) instruction manual, respectively. The library was amplified using a Chromium Single Cell Human TCR amplification kit (Cat. No.: 1000252) and a Chromium Single Cell Human BCR amplification kit (Cat. No.: 1000253) according to the product instructions.

Spatial transcriptome sequencing

In order to perform spatial transcriptome sequencing, the primary sample was directly obtained from the operating room, and test sample was generated from this primary sample. Specimens were dissociated into single cells according to protocols. The fresh frozen tissues for ST were stored at −80 °C and embedded in OCT (Sakura, Alphen aan den Rijn, Netherlands) before cryosectioning for RNA extraction. Each tissue was cut with thicknesses of 10 µm in the cryostat (Leica CM1950, Germany) and mounted onto ST microarrays. For processing, the tissue was dehydrated with isopropanol for 1 min and staining with H&E. Bright-field images were obtained by a whole slide scanner (Pannoramic MIDI FL, 3DHISTECH, Hungary) at 20× resolution. H&E-stained sections of each sample were carefully reviewed by two experienced pathologists to confirm the pathology.

Library preparation slides used were purchased from the ST team (<https://www.spatialtranscriptomics.com>). Tissue sections were subjected to Visium Spatial Gene Expression Reagent Kits (10× Genomics, Pleasanton, CA) for library preparation according to the standard protocols. Libraries were prepared according to the Visium Spatial Gene Expression User Guide (CG000239, Rev B, 10× Genomics). The constructed library was sequenced on Illumina (San Diego, USA) NovaSeq 6000 using a paired-end 150 bp.

The FASTQ files were processed and aligned to GRCh38 human reference genome using Space Ranger software (version 2.0.1) from 10× Genomics, with unique molecular identifier (UMI) counts summarized for each barcode. To distinguish tissue overlaying spots from the background, tissue overlaying spots were detected according to the images. The filtered UMI count matrix was then analyzed using Seurat⁴⁹ (version 4.1.0) R package.

Sctransform⁵⁰ was used to normalize data and identify top 3000 highly variable genes (HVGs). Principal component analysis (PCA) was conducted to reduce dimensionality on the log transformed gene-barcode matrices of top variable genes. Graph-based clustering was performed to cluster cells according to their gene expression profile with the FindClusters function. Cells were visualized using a 2-dimensional Uniform Manifold Approximation and Projection (UMAP) algorithm with the RunUMAP function. The FindAllMarkers function (test.use = bimod) was used to identify marker genes of each cluster. Differentially expressed genes (DEGs) were selected using the Seurat⁴⁹ function FindMarkers (test.use = presto). P value < 0.05 and |log2foldchange|

> 0.58 was set as the threshold for significantly differential expression. GO enrichment and KEGG pathway enrichment analysis of DEGs were respectively performed using R (version 4.0.3) based on the hypergeometric distribution. The sequencing and bioinformatics analysis were provided by OE Biotech Co., Ltd. (Shanghai, China).

Immunofluorescence staining of liver cancer biopsies

Biopsies from a 4-patient cohort before and after OV treatment (in the 3 days after the second OV treatment cycle) were processed for multiplex immunofluorescence (mIF) staining. The formalin-fixed paraffin-embedded (FFPE) biopsies were sliced into 3- μ m sections and placed onto slides. The FFPE slides were dewaxed in xylene and rehydrated through a graded series of ethanol solutions (100%, 95%, 85%, 75%), followed by rehydration using distilled water. Next, the slides were fixed in 10% neutral buffered formalin for 20 min, immersed in sodium citrate antigen retrieval buffer (pH 6.0), and microwaved for 45 s at 100% power and 15 min at 25% power. After cooling at room temperature, the slides were transferred to TBST (pH 7.4) and shaken for 5 min on the decolorizing shaker (three times). Subsequently, the slides were covered with blocking buffer for 10 min, then incubated with primary antibodies to detect the relevant protein marker at room temperature for 1 h or 4°C overnight. The slides were washed three times using TBST, and incubated for 10 min with Opal Polymer HRP Ms+Rb for 10 min. If necessary, the above steps were repeated until all targets of interest had been detected. The slides were stained for 5 min using 4',6-diamidino-2-phenylindole at room temperature and analyzed using a multichannel fluorescence scanner.

805

806 **Cell culture**

807 The Hep3B and Vero cell lines were from American Type Culture Collection (Manassas,
808 VA, USA). Cells were cultured in an incubator at 37°C with 5% CO₂ (Hep3B: minimum
809 essential medium [MEM], 11095-080, Gibco, Grand Island, NY, USA; Vero:
810 Dulbecco's modified Eagle's medium [DMEM], SH30243.01, HyClone, Logan, UT,
811 USA) supplemented with 10% fetal bovine serum (FBS, 16000-044; Gibco) and 1%
812 penicillin-streptomycin solution (P/S) (CR-15140; Cienry, Zhejiang, China).

813

814 **Virus infection and replication assay**

815 Well-proliferated Hep3B cells were added to 96-well plates and adhered. Then, gradient
816 dilutions of VG161 and/or the respective anti-hepatitis B virus (HBV) agents were
817 prepared, with eight gradients per donor. Additionally, medium control and cell control
818 groups (containing an equal number of cells without viral infection) were established.
819 Extended Data Tables 6 and 7 present the specific concentration gradients and
820 concentrations of each anti-HBV agent used with VG161, respectively. After 65-h
821 infection, 20 µL Cell Counting Kit-8 (CCK-8) reagent was added to each well and
822 incubated for 2 h at 37°C before the absorbance was read at 450 nm. The mean medium
823 control and cell control group values were used as the cell viability 0% and 100% values,
824 respectively. Cell viability was determined using the mean values of the experimental
825 groups with different multiplicities of infection (MOI) as follows: experimental group
826 cell viability (%) = (experimental group - medium control group) / (cell control group -

medium control group) $\times 100\%$. The experimental groups and their cell viability values underwent 4-parameter curve fitting using GraphPad Prism 8.0.

Animal experiments

Female NCG and NOG mice (between 5 and 6 weeks old) were from Gempharmatech Co., Ltd. (Jiangsu, China). The Zhejiang Center of Laboratory Animals Institutional Animal Care and Use Committee (IACUC) approved all animal experiments (Approval Nos. ZJCLA-IACUC-20030088 and 20070037). Tumor volume was measured every 2-3 days and calculated as follows: $0.5 \times \text{long diameter} \times \text{short diameter}^2$.

Hep3B cells (5×10^6) were implanted subcutaneously into the left axilla of 6-week-old NCG mice. The mice were randomized into groups ($n = 3$ per group) and gavaged with entecavir (10% suspension, Bristol-Myers Squibb Co., Ltd.) or saline when the mean tumor volume was 80-100 mm³ (14 days post-inoculation). The tumors were injected intratumorally with VG161 3 days after the gavage.

The Hep3B tumor-bearing mice were injected with 1×10^7 PFU VG161 and gavaged with saline or entecavir. After 0.5, 1, 2, 3, and 7 days, the mice were killed, and the spleen, lung, liver, heart, kidneys, blood, and lesion tissues were harvested. Total DNA was extracted using a DNeasy Blood and Tissue Kit (69504; Qiagen, Venlo, The Netherlands) according to the manufacturer's protocol. The virus genome copy number was quantified using qPCR (7900HT; Applied Biosystems) targeting the OV IL-15R α 1 gene. All reactions included negative controls and were run in triplicate. The data were analyzed using SPSS v.2.4 (Applied Biosystems).

849

850 **Statistical analysis**

851 The demographic and baseline characteristics and safety analysis included all patients
852 enrolled (dose escalation and expansion cohorts). The tumor response analysis included
853 all patients with HCC, excluding three patients who withdrew their informed consent
854 form (ICF) before the initial tumor assessment and two patients who did not respond to
855 first-line treatment. The PFS and OS analyses excluded one patient who died from
856 COVID-19.

857 The response rate and corresponding ORR and DCR 95% confidence intervals (CI)
858 were calculated using the Clopper-Pearson method. The PFS and OS were analyzed
859 using Kaplan–Meier (KM) methods. Median values and their 95%CI were determined
860 using the Brookmeyer and Crowley method with log–log transformation. Survival rates
861 and their 95%CI were calculated using the Greenwood formula. Subgroups were
862 compared according to the data type using the *t*-test, Fisher’s exact test, chi-square test,
863 or log-rank test.

864

865 **Reporting summary**

866 Further information on the research design is available in the Nature Portfolio
867 Reporting Summary linked to this article.

868

869 **Data availability**

870 The study protocol is available in the Supplementary Information. The datasets from

the clinical trial can be requested 12 months after the publication of this article. Researchers wishing to access raw and analyzed data should email the corresponding author (T.L.), clearly stating the research purpose. Requests will be reviewed by the institutional review board, considering the risk of patient reidentification, and a response can be expected within 14 days. Individual deidentified data of the participants will be available to approved eligible applicants and investigators after signing a data access agreement. Source data are provided with this paper.

Code availability

No custom code or mathematical algorithm was used, SAS software (version 9.4), R and Python software was used for data analysis ('Seurat' package, 'harmony' package, 'scRepertoire' package, 'DESeq2' package, 'survival' package, 'survminer' package, 'glmnet' package, 'survivalROC' package, 'rms' package, 'timeROC' package, 'SPOTlight' package, 'ggplot2' package, 'stlearn' package).

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922

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Author contributions

Y.S., Q.T., R.Z., and T.L. designed the clinical trial. Y.S., X.B., Q.Z., X.L., S.G., G.S., Y.W., T.F., Y.L., L.W., X.S., W.C., Y.Y., Y.C., and X.L. were responsible for patient treatment and management. X.J. and G.W. performed ultrasound-guided puncture administration. Z.Z., J.S., and M.C. recruited patients and collected data. Y.S., A.Q., L.X., Y.Q., W.B., S.R., R.Z., and M.H. conducted statistical analysis. Z.Z., W.J., Z.W., Z.Y., S.G., D.L., F.W., and J.D. were responsible for the basic research work. W.S., M.S., and Y.M. performed bioinformatics analysis. Y.S. and T.L. wrote the manuscript. All authors read and approved the final draft of the paper.

Competing interests

CNBG-Virogin Biotech (Shanghai) Co., Ltd. owns the patent rights for VG161 in mainland China. Virogin Biotech Co., Ltd. owns the patent rights for VG161 outside mainland China. The authors declare no competing interests.