Chimeric Antigen Receptor-Glypican-3 T-Cell Therapy for Advanced Hepatocellular Carcinoma: Results of Phase I Trials

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ABSTRACT

Purpose: Our preclinical studies demonstrated the potential of chimeric antigen receptor (CAR)-glypican-3 (GPC3) T-cell therapy for hepatocellular carcinoma (HCC). We report herein the first published results of CAR-GPC3 T-cell therapy for HCC.

Patients and Methods: In two prospective phase I studies, adult patients with advanced GPC3^{+} HCC (Child-Pugh A) received autologous CAR-GPC3 T-cell therapy following cyclophosphamide- and fludarabine-induced lymphodepletion. The primary objective was to assess the treatment's safety. Adverse events were graded using the Common Terminology Criteria for Adverse Events (version 4.03). Tumor responses were evaluated using the RECIST (version 1.1).

Results: A total of 13 patients received a median of 19.9×10^8 CAR-GPC3 T cells by a data cutoff date of July 24, 2019. We observed pyrexia, decreased lymphocyte count, and cytokine release syndrome (CRS) in 13, 12, and nine patients, respectively. CRS (grade 1/2) was reversible in eight patients. One patient experienced grade 5 CRS. No patients had grade 3/4 neurotoxicity. The overall survival rates at 3 years, 1 year, and 6 months were 10.5%, 42.0%, and 50.3%, respectively, according to the Kaplan–Meier method. We confirmed two partial responses. One patient with sustained stable disease was alive after 44.2 months. CAR T-cell expansion tended to be positively associated with tumor response.

Conclusions: This report demonstrated the initial safety profile of CAR-GPC3 T-cell therapy. We observed early signs of antitumor activity of CAR-GPC3 T cells in patients with advanced HCC.

Introduction

Hepatocellular carcinoma (HCC) is the most common histologic subtype of liver cancer, which is the sixth most common cancer and the fourth leading cause of cancer-related death worldwide (1). Half of all liver cancer cases and deaths are estimated to occur in China (2). In the United States, the incidence of liver cancer has more than tripled since 1980 (3).

Considerable challenges exist in the clinical management of HCC. Only 15% to 20% of HCC cases are diagnosed at an early stage that may be suitable for curative treatment, such as surgical resection, liver transplantation, local ablation with percutaneous ethanol injection, microwave ablation, and radiofrequency ablation (4–7). Meanwhile, the majority of patients with HCC have underlying chronic liver disease, and resection in this population is fraught with the potential for complications. Patients with intermediate-stage disease may benefit from local therapies such as transarterial chemoembolization. However, relapse is a frequent and expected event after systemic and local therapies (8). Also, many patients are diagnosed with unresectable advanced-stage HCC, with macroscopic vascular invasion and extrahepatic spread. Sorafenib, a targeted kinase inhibitor, was the first systemic therapeutic approved by the FDA for HCC based on improvement in overall survival (OS) duration by about 3 months (9, 10). In addition, regorafenib and two programmed cell death protein 1 (PD-1) inhibitors, pembrolizumab and nivolumab, were recently approved for HCC treatment in patients who experienced progression after taking sorafenib (11–13). Most recently, lenvatinib, cabozantinib, and ramucirumab were approved for patients with HCC who had progression after sorafenib treatment (14–17). The approval of anti–PD-1 inhibitors, pembrolizumab and nivolumab, demonstrates that HCC is an immunosensitive tumor. Furthermore, tremelimumab, an anti-cytotoxic T-lymphocyte–associated protein 4 (CTL-4) antibody, produced response rates of 17% to 26% in patients with HCC (18, 19). A pilot randomized trial of perioperative immunotherapy with nivolumab and ipilimumab (anti–CTLA-4) for resectable HCC is ongoing, with preliminary results demonstrating good antitumor activity (20). Despite progress in available therapies, effective systemic treatment options for HCC are still limited. Thus, its 5-year survival rate is a dismal 18%; this rate decreases to 11% and 5% if cancer has spread into surrounding tissues and distant parts of the body, respectively (21).

In recent years, chimeric antigen receptor (CAR) T-cell therapy has achieved significant efficacy in the treatment of hematologic

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Translational Relevance

Glypican-3 (GPC3) is a member of the heparan sulfate proteoglycan family that may play a role in the control of cell division and growth regulation. GPC3 positive immunostaining can differentiate hepatocellular carcinoma (HCC) from dysplastic changes in cirrhotic livers. Recent studies demonstrated that greater GPC3 expression in tumor cells is associated with a worse prognosis for HCC. This observation provides a rationale for chimeric antigen receptor (CAR)-GPC3 T-cell therapy in advanced HCC. This report demonstrated that CAR-GPC3 T-cell therapy was feasible in patients with HCC. Special attention should be given to patients with rapidly progressive disease and/or heavy tumor burden. Early signals of antitumor activity were observed. This publication is the first report of CAR-GPC3 T-cell therapy in patients with advanced HCC.

malignancies (22–26). Although a breakthrough in disease control using CAR T-cell therapy for solid tumors has yet to be achieved, researchers have observed encouraging antitumor activity in early-phase clinical trials of therapies targeting IL13Rα2 (27), mesothelin (28), and claudin18.2 (29, 30), suggesting the feasibility of CAR T-cell therapy for solid tumors.

Glypican-3 (GPC3) is a member of the heparan sulfate proteoglycan family and attaches to cell surfaces via a glycosylphosphatidylinositol anchor. Recent studies demonstrated that GPC3 may be a prognostic marker for HCC, with greater GPC3 expression in tumor cells associated with worse prognosis (31). GPC3 is a molecule that is not fully understood in the role of proliferation and suppression of cell growth in normal tissues and abnormal or cancerous tissues. Of note, our previous studies demonstrated that GPC3 was highly expressed in HCC and squamous non–small cell lung cancer but showed no expression in the kidney and gastric glands (32, 33). These findings suggest that GPC3 is a rational immunotherapeutic target for HCC. Indeed, anti-GPC3 mAbs had good safety profiles in previous studies (34, 35), although significant clinical benefit has yet to be established in phase II clinical trials. Recently, we demonstrated that GPC3-targeted CAR T cells could eliminate $GPC3^+$ HCC cells *in vitro* and eradicate $GPC3^+$ HCC tumor xenografts in mice (36–38). Therefore, in the present prospective phase I studies, we explored the safety and potential efficacy of CAR-GPC3 T-cell therapy in adult Chinese patients with advanced $GPC3^+$ HCC. We hypothesized that CAR-GPC3 T cells approach was feasible and could be safely tolerated among patients with advanced HCC.

Patients and Methods

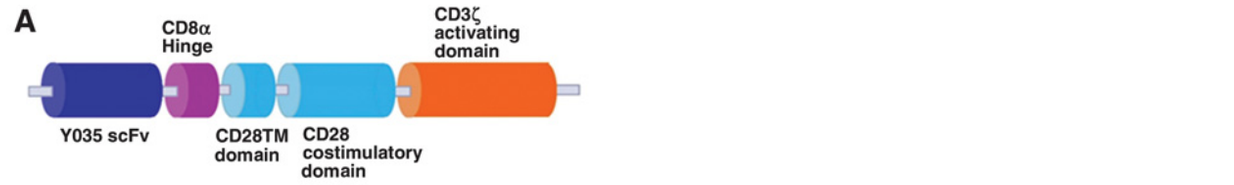
Clinical trial design and patients

Here we report two sequential phase I studies: NCT02395250 (study 1, principal investigator, Z. Li) and NCT03146234 (study 2, principal investigator, B. Zhai). Both studies were approved by the Ethics Committee of Renji Hospital, Shanghai Jiao Tong University School of Medicine. Both trials were performed in accordance with the principles of the Declaration of Helsinki. All patients provided written informed consent to participate. Pooling the data allowed for a more robust analysis with an appropriate number of patients to evaluate pharmacology dynamics and clinical responses.

Patients who were screened for GPC3 expression must have had relapsed HCC at least twice within 2 years on standard HCC therapy per *Guidelines for Diagnosis and Treatment of Primary Liver Cancer in China* (2011 edition) (39). As described previously (33), their GPC3 expression status was determined via IHC staining of fresh tumor samples or archived samples collected at local laboratories within 6 months before enrollment. GPC3 expression was evaluated using a 4-point scale: 0, no GPC3 expression; 1+, weak expression; 2+, medium expression; 3+, strong expression (Supplementary Materials and Methods). Eligibility criteria included one or more measurable lesions per the RECIST (version 1.1), a Child-Pugh score of A or B with a score of 7, and an Eastern Cooperative Oncology Group (ECOG) performance score of up to 1. The patients must have had reasonable hematologic cell counts and sufficient renal and hepatic function. Exclusion criteria included active ascites requiring treatment, human immunodeficiency virus infection, and the presence of an autoimmune disease. A full description of the study design and eligibility criteria is provided in the study protocols (Supplementary Materials and Methods).

Patients who met all screening criteria underwent leukapheresis to obtain peripheral blood mononuclear cells (PBMC) for the generation of autologous CAR-GPC3 T cells. As described previously (36), CAR-GPC3 T cells (product code Y035) consisted of a humanized anti-GPC3 single-chain variable fragment, CD8α hinge domain, CD8α transmembrane domain, CD28 intracellular domain, and CD3ζ intracellular signaling domain that were cloned into a lentiviral backbone (**Fig. 1A**). After the CAR-GPC3 cells were manufactured, the patients were admitted to the hospital. Two to six days before CAR-GPC3 T-cell infusion, patients underwent lymphodepletion (**Fig. 1B**). In study 1, the lymphodepletion regimen used either (1) cyclophosphamide (Cy) 500 to 1,000 mg/m^2/day for 1 to 2 days, or (2) the combination of Cy (500 mg/m^2/day for 1–3 days) and fludarabine (Flu, 25–30 mg/m^2/day for 2–4 days). In study 2, all patients received the combination regimen of Cy (500 mg/m^2/day for 1–2 days) and Flu (20–25 mg/m^2/day for 3–4 days). The lymphodepletion regimen could be adjusted by the treating physician according to patient's disease condition (Supplementary Table S1). One subject (patient P5) was determined to be unfit for lymphodepletion due to poor clinical condition, with a heavy tumor burden and extremely elevated α-fetoprotein (AFP) level of 60,500 ng/mL.

After lymphodepletion, patients received a cycle of Y035 CAR-GPC3 T-cell therapy under observation and were not discharged until their absolute neutrophil counts recovered to at least 500 cells/mm^3. In study 1, GPC3-CAR T cells were administrated from a starting split dose (1×10^5 CAR positive cells/kg) and gradually increased by two- to threefold until the final total dose of the split infusion was reached. The maximum dose was 2×10^9 CAR positive cells/kg if sufficient cells could be manufactured. The maximum dose would be de-escalated if there was a dose-limiting toxicity (DLT) related to the CAR-T treatment. The total dose could be administrated in two to nine sequential split infusions over the treatment cycle. In study 2, manufacturing capabilities were optimized, and all patients (P9-P13) were treated with the fixed dose recommended from study 1 (20.0×10^8 CAR positive cells). Patient P1 received two cycles of Y035 CAR-GPC3 T-cell infusion. The dose of the first cycle was split into six infusions given over 13 days to ensure safety. The dose of the first split infusion was 0.08×10^8 cells (1×10^5 cells/kg) and the remaining split doses were gradually increased by two to three times until the final split dose of 20.0×10^8 cells was reached. A total dose of 40.7×10^8 Y035 CAR-GPC3 T cells were administered in cycle one. The remaining 11.1×10^8 Y035 CAR-GPC3 T cells were administered in cycle two. The interval between the two cycles of Y035 CAR-GPC3 T-cell infusion in patient P1 was approximately 6 weeks. Patients P2 to



	Study 1	Study 2
Subject ID	P1–P8	P9–P13
Lymphodepletion regimen	(1) Cy 500–1,000 mg/m²/day for 1–2 days; (2) Cy (500 mg/m²/day for 1–3 days) + Flu (25–30 mg/m²/day for 2–4 days)	Cy (500 mg/m²/day for 1–2 days) + Flu (20–25 mg/m²/day for 3–4 days)
CAR-GPC3 T cell dose (per cycle)	Starting dose: 1×10^5/kg interpatient dose escalation. The maximum permissible dose level of 2×10^9/kg of CAR+ T cells was not reached.	One dose of 20.0×10^8 of CAR+ T cells was administered. The maximum tolerated dose was not reached.

† Hospitalization period includes lymphodepletion and CAR T infusion as well as postinfusion in-patient monitoring.
Cy, Cyclophosphamide; Flu, Fludarabine.

Figure 1.

CAR-GPC3 construct and study design. **A,** The modular composition of Y035 CAR-GPC3. scFv, single-chain variable fragment. **B,** Protocol schedule for screening, manufacturing autologous CAR-GPC3 T cells, and lymphodepletion followed by infusion of CAR-GPC3 T cells and follow-up. The differences in the lymphodepletion regimen and T-cell dose between the two studies are listed.

P8 received one cycle of Y035 CAR-GPC3 with a mean of 23.2×10^8 cells (median, 9.6×10^8 cells; range, $7.0–92.5 \times 10^8$ cells; **Table 1**).

After T-cell infusion, each patient's CAR T-cell DNA copy number and cytokine levels were monitored. The incidence and severity of adverse events (AEs) were graded using the Common Terminology Criteria for Adverse Events (version 4.03). Special attention was given to CAR T-cell–related infusion reactions, cytokine release syndrome (CRS), and preconditioning-related infectious diseases. Early diagnosis and management of CRS were based on Lee's criteria (40). Tumor response was assessed using RECIST (version 1.1). CT or MRI was performed during follow-up. Patients were evaluated every 4 to 8 weeks for the first 6 months and then about every 3 months per the standard of care.

qRT-PCR analysis of CAR-GPC3 DNA copy numbers

Real-time fluorescent qPCR was applied to determine the CAR-GPC3 DNA copy numbers in peripheral blood as described previously (41). Genomic DNA was extracted from patient PBMCs using a QIAamp DNA Blood Midi Kit (QIAGEN) before and after CAR T-cell infusion. The forward primer 5′-CCAGCTGCTGATCTACAAGG-3′, reverse primer 5′-CAGTAGTACACGCCCACGTC-3′, and TaqMan probe 5′-FAM-CGGCACCGACTTCACCCTGA-TAMRA-3′ were used in the qPCR assay.

Immunological assays to measure blood concentrations of cytokines and tumor biomarkers

Both plasma and serum samples were collected from patients before and at different time points after CAR T-cell infusion. Specific circulating cytokines testing was performed on the same type of samples. The plasma concentrations of IL6, IL12p70, IFNγ, regulated upon activation, normal T cell expressed, and secreted (RANTES), macrophage inflammatory protein (MIP)-1β, and monocyte chemoattractant protein (MCP)-1 were measured using cytometric bead array (CBA) according to the manufacturer's instructions (BD Biosciences; catalog numbers 558276, 558283, 560111, 558324, 558288, and 558287, respectively). The serum concentration of C-reactive protein (CRP) was measured using a high-sensitivity CRP assay with a BN II System (Siemens Healthineers). The serum AFP concentrations were measured using a chemiluminescent immunoassay according to the manufacturer's instructions (ARCHITECT i2000 chemiluminescence immunoassay analyzer, automated; Abbott Diagnostics).

Table 1. Patient characteristics, Y035 CAR-GPC3 T-cell doses, and clinical outcomes.

Subject ID[a]	Age (years)	Sex	ECOG score	GPC3 IHC staining intensity score	HCC history (years)	BCLC stage	MVI	Metastasis	Cirrhosis	Therapy cycle	CAR^+ T-cell dose (cells)	CAR^+ T-cell dose[b] (cells/kg)	Response	OS duration[c] (days)	PFS duration (days)
P1	54	M	0	3+	0.6	C	Yes	Yes	No	1	40.7×10^8	49.6×10^6	SD	1,326	204
										2	11.1×10^8	13.5×10^6			
P2	34	F	1	3+	0.8	C	No	Yes	Yes	1	17.4×10^8	38.7×10^6	NE	95	95
P3	50	M	0	3+	2.2	C	No	Yes	No	1	7.8×10^8	10.8×10^6	PR	615	111
P4	70	M	1	1+	10.9	C	No	Yes	No	1	92.5×10^8	146.9×10^6	SD	601	81
P5	59	M	1	3+	2.0	D	No	Yes	No	1	9.6×10^8	15.3×10^6	PD	29	29
P6	39	M	0	3+	0.4	C	No	No	No	1	19.7×10^8	41.5×10^6	PD	693	33
P7	44	F	1	1+	1.5	C	No	No	No	1	8.7×10^8	15.3×10^6	NE[d]	278	18[d]
P8	46	M	1	3+	7.1	C	No	Yes	No	1	7.0×10^8	15.5×10^6	NE[d]	127	40[d]
P9	51	M	1	3+	2.2	C	No	Yes	No	1	20.0×10^8	26.7×10^6	PD	134	34
P10	51	M	1	3+	0.8	C	Yes	Yes	No	1	20.0×10^8	37.7×10^6	PD	48	13
P11	68	M	1	2+	6.1	B	No	No	Yes	1	20.0×10^8	30.8×10^6	PD	132	34
P12	53	M	1	3+	2.3	B	No	Yes	Yes	1	20.0×10^8	44.4×10^6	NE	24	24
P13	51	M	0	3+	3.7	B	No	No	No	1	20.0×10^8	26.3×10^6	PR	385	99

Abbreviations: BCLC, Barcelona Clinic Liver Cancer; F, female; ID, identification; M, male; MVI, macrovascular invasion; NE, not evaluable; PD, progressive disease.

[a]P1 to P8 were enrolled in study 1 and P9 to P13 were enrolled in study 2. P3 and P9 was same patient who enrolled twice. After the initial enrolment in study 1 (indicated by P3), the patient withdrew from study 1 for about 1 year and then enrolled in study 2 (indicated by P9).

[b]Calculated on the basis of body weight.

[c]The data cutoff date was July 24, 2019. The OS values for the patients who were alive on that date (P1 and P13) were censored.

[d]Clinical progression in P7 and P8.

Statistical analysis

Patients in study 1 and study 2 were analyzed together. Descriptive statistics consisted of medians with ranges and means with SDs for continuous variables and counts and percentages for categorical variables. AE terms were coded using the Medical Dictionary for Regulatory Activities (version 21.1). Analyses of the association of DNA copy numbers or cytokine levels with CRS and time points of tumor response were performed using SAS software (version 9.4; SAS Institute). Progression-free survival (PFS) and OS were analyzed using the Kaplan–Meier method. SAS software (version 9.4; SAS Institute) was used to calculate pharmacokinetic parameters using trapezoidal rule.

Results

Patient characteristics

A total of 13 patients with HCC received Y035 CAR-GPC3 T-cell therapy from December 2015 to August 2018. P1 to P8 were enrolled in study 1. P9 to P13 were enrolled in study 2. The patients (11 male and two female) had a median age of 51 years (range, 34–70 years; **Table 1**). All patient tumors were positive for GPC3 according to IHC staining, including 10 patients with a staining intensity score of 3+. All patients had Child-Pugh class A disease and 3 patients had cirrhosis. Ten patients had extrahepatic disease, 9 had longer than a 1-year HCC history, and 10 had Barcelona Clinic Liver Cancer stage C/D disease. Patients had received prior surgical resection, local therapy, or systemic therapy (Supplementary Table S2). All patients had a history of hepatitis B viral infection and 5 patients received entecavir antiviral treatment (Supplementary Table S3).

Characteristics of Y035 CAR-GPC3 T cells

We successfully generated Y035 CAR-GPC3 T cells for all 13 patients. The median time to manufacture the cell products for clinical use was 12 days (range, 10–13 days). A median of 64.4% T cells (range, 41.4–88.4%) expressed CAR-GPC3. Immunophenotypic analysis demonstrated that more than 95.8% of transduced T cells were $CD3^+$ [mean, 98.6% (range, 95.8–99.6%)], and both $CD8^+$ and $CD4^+$ subsets were present in all Y035 CAR T-cell products (data not shown). Y035 CAR T-cell products were predominantly terminally differentiated effector memory T cells [$CD45RA^+/CCR7^-$; mean, 78.2% (range, 42.3–94.1%)], and effector memory T cells [$CD45RA^-CCR7^-$; mean, 14.1% (range, 0.8–47.4%); **Fig. 2A–C**].

Adverse effects

Y035 CAR-GPC3 T-cell therapy was generally tolerable in patients who had low tumor burdens and were in good clinical condition, even with doses greater than 20.0×10^8 CAR-positive cells. All but one patient experienced an expected transient grade 3/4 decrease in lymphocyte count resulting from chemotherapy-induced lymphodepletion. We observed CRS in nine patients (**Table 2**; Supplementary Table S4). No patients experienced grade 3/4 neurotoxicity and no patients experienced CAR T-cell–related infusion reactions.

Persistence of Y035 CAR-GPC3 T cells in peripheral blood

The CAR-GPC3 vector copy number was closely monitored in the first 2 weeks after the initial infusion, weekly in the first month after the last infusion, and monthly thereafter. The median CAR-GPC3 DNA copy number in the peripheral blood of all patients increased rapidly, reaching a peak of 360.4 copies/μg

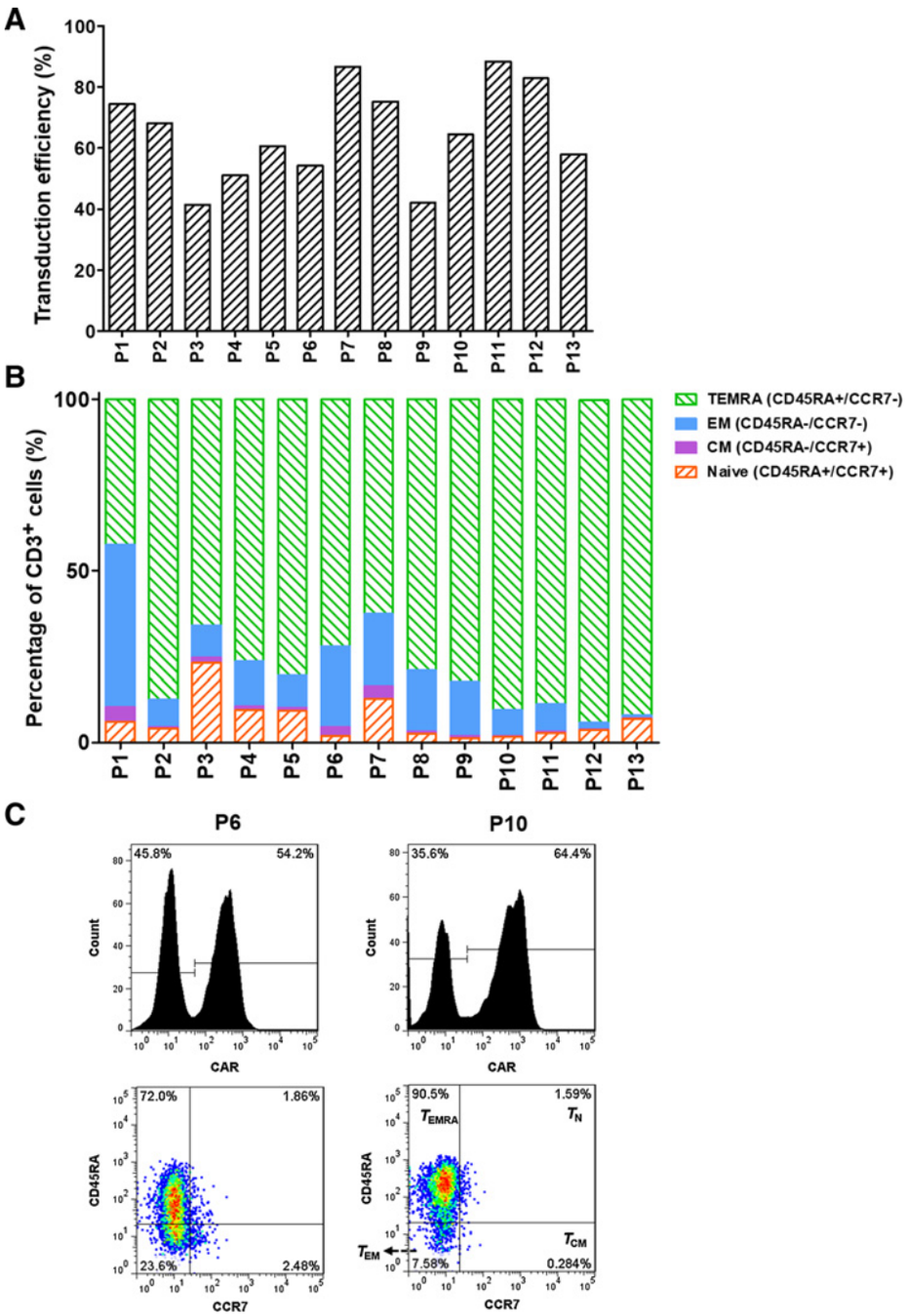


Figure 2.

Characteristics of Y035 CAR-GPC3 T cells, which were successfully generated for all patients. **A,** CAR-GPC3 expression in the T cells in the 13 patients with HCC. A median of 64.4% (range, 41.4–88.4%) of T cells expressed CAR-GPC3 according to fluorescence-activated cell sorting analysis. **B,** Subset composition of Y035 CAR-GPC3 T-cell products infused in all patients. **C**, Representative histograms and dot plots of infused Y035 CAR-GPC3 T-cell products (patients P6 and P10). CM, central memory; EM, effector memory; TEMRA, terminally differentiated effector memory T cells.

genomic DNA (range, 28.0–23,358.0 copies/μg genomic DNA) after a median period of 10.5 days (mean, 13.8 days) and lasting for a median duration of 19.5 days (mean, 34.4 days; **Fig. 3A**). The AUCs for the CAR-GPC3 DNA copy numbers in two patients who had objective responses tended to be higher than those in the other 10 patients (**Fig. 3B**), with median AUCs of 98,016.0 and 5,423.1, respectively ($P = 0.0606$). In the two responders, we observed CAR T-cell DNA copy number peaks about 2 weeks after Y035 CAR-GPC3 T-cell infusion and before the first partial response (PR; **Fig. 3C**). The longest CAR T-cell DNA copy number duration was about 140 days.

Cytokine profile and CRS

We monitored patient serum levels of 12 cytokines during the studies. The median fold increase in IFNγ and IL6 level over baseline was 68.2 and 34.6, respectively. The median peak value of IFNγ and IL6 level postinfusion was 3.98 and 172.82 pg/mL, respectively (**Fig. 3D–E**). The levels of CRP, IL6, RANTES, and MCP-1 in patient P3 peaked at the same time as the patient's body temperature increase, which was consistent with clinical observations of CRS in patients who received Y035 T-cell infusion. In addition, we observed IL6, IL10, IL15, and IFNγ peaks before the CRS event in patient P13 and significant decreases in these levels after the event (**Fig. 3F**).

Table 2. Treatment-emergent AEs in patients who received Y035 CAR-GPC3 T-cell infusions.

Subject ID	Cytopenia (AE grade)	CRS-related events (AE grade)	CRS grade	High-dose steroids	Use of IL-6R inhibitor
P1	WBC decreased (2), platelet count decreased (2), lymphocyte count decreased (4)	Pyrexia (2),APTT increased (1)	1	No	No
P2	Lymphocyte count decreased (4)	AST level increased (1), APTT increased (1)	1	No	No
P3	WBC decreased (2), lymphocyte count decreased (4)	Pyrexia (2), bilirubin level increased (2)	1	No	No
P4	Lymphocyte count decreased (3)	Pyrexia (1), AST level increased (1), ALT level increased (1)	1	No	No
P5	Platelet count decreased (2)	Not applicable	0	No	No
P6	WBC decreased (4), lymphocyte count decreased (4)	Not applicable	0	No	No
P7	Platelet count decreased (1), lymphocyte count decreased (4)	Not applicable	0	No	No
P8	Lymphocyte count decreased (4)	Not applicable	0	No	No
P9	WBC decreased (2), platelet count decreased (3), lymphocyte count decreased (4)	Pyrexia (2), APTT increased (1)	1	Yes	No
P10	Platelet count decreased (3), lymphocyte count decreased (4)	Pyrexia (3), renal impairment (1), AST level increased (2), ALT level increased (1), bilirubin level increased (4), APTT increased (1)	2	Yes	No
P11	WBC decreased (2), neutrophil count decreased (2), platelet count decreased (2), lymphocyte count decreased (4)	Pyrexia (2), AST level increased (1), bilirubin level increased (3), APTT increased (1)	2	No	No
P12	Platelet count decreased (4), lymphocyte count decreased (4)	AST level increased (2), ALT level increased (1), bilirubin level increased (4), APTT increased (1)	5	Yes	Yes
P13	Neutrophil count decreased (2), platelet count decreased (3), lymphocyte count decreased (4)	Pyrexia (2), hypotension (2), bilirubin level increased (2), APTT increased (1)	2	Yes	Yes

Note: AE terms were coded using the Medical Dictionary for Regulatory Activities (version 21.1). AE grading was assessed using the Common Terminology Criteria for Adverse Events (version 4.03). CRS grading was assessed using Lee's criteria (40).

Abbreviations: ALT, alanine transaminase; APTT, activated partial thromboplastin time; AST, aspartate aminotransferase; ID, identification; WBC, white blood cell count.

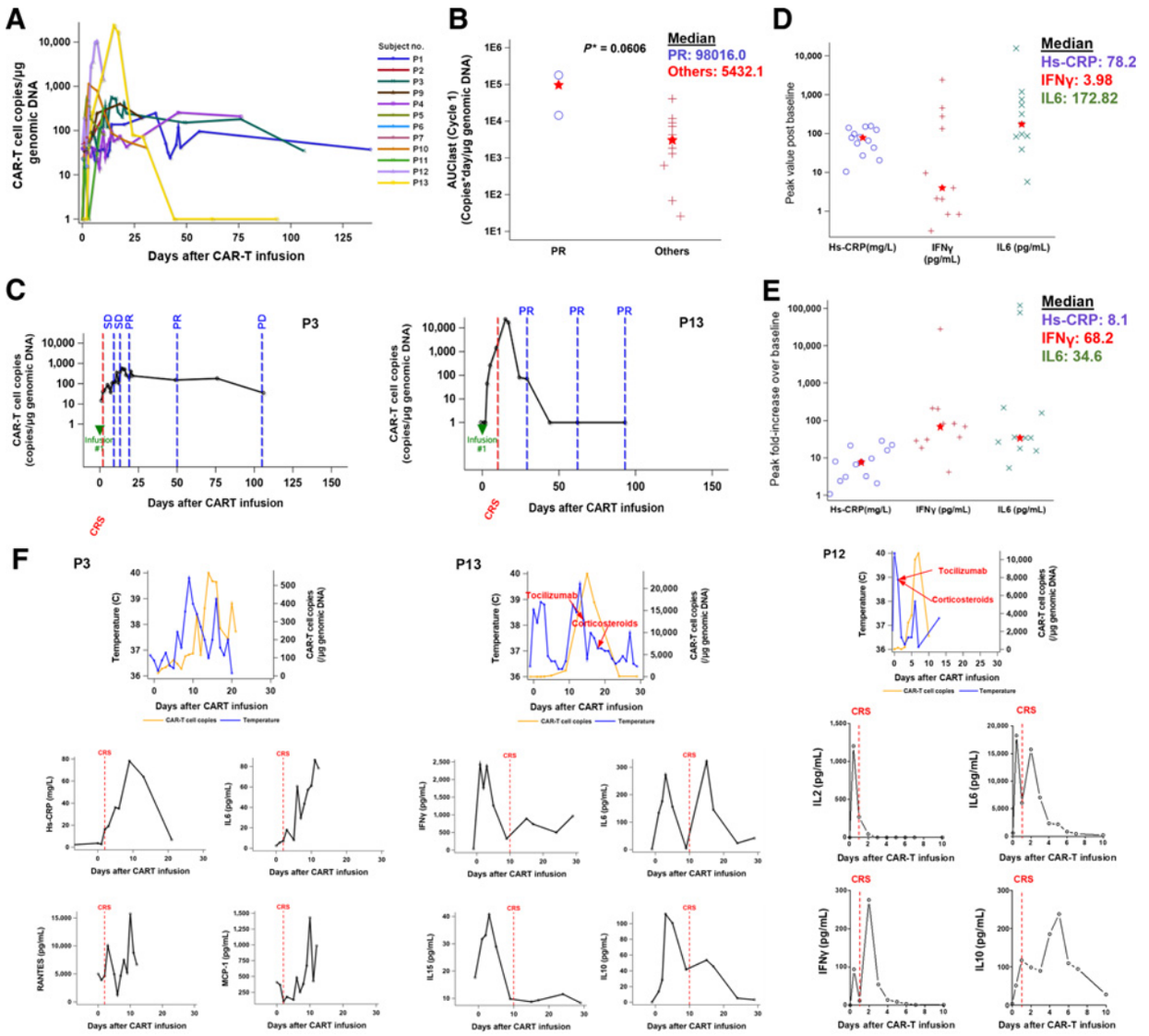


Figure 3.

Persistence of CAR-GPC3 T cells as determined by DNA copy numbers, and cytokine levels in the patients with HCC. **A,** Persistence of CAR-GPC3 T cells in patients with HCC assessed using qRT-PCR to determine the expression of CAR-GPC3 transgenes in peripheral blood genomic DNA. Semi-log CAR-GPC3 copy numbers were plotted over time. One patient (P8) exhibited clinical disease progression after the treatment and withdrew consent for CAR-GPC3 copy-number testing. **B,** The association of CAR-GPC3 DNA copy number with tumor response. Copy numbers in the two responders (P3 and P13) were compared with those in the other patients. The area under the CAR-GPC3 T-cell copies–time curve from time zero to time of last measurable value (AUC_{last} for the CAR-GPC3 copy number) was measured after the first cycle of CAR-GPC3 T-cell infusion. The median CAR-GPC3 DNA copy number as calculated according to the AUC_{last} tended to be higher in P3 and P13 than in the 11 other patients ($P = 0.0606$). Circles indicate individual patients. **C,** Semi-log CAR-GPC3 DNA copy numbers in patients P3 and P13 plotted with CRS (red) and time points of tumor responses (blue). Each CAR T-cell infusion is marked by a green triangle. PD, progressive disease. **D,** The peak levels of three representative cytokines after the first cycle of CAR-GPC3 T-cell infusion in all patients. Hs-CRP, high-sensitivity CRP. **E,** The peak fold increases in the levels of three representative cytokines after the first cycle of CAR-GPC3 T-cell infusion in all patients. **F,** Results of cytokine analyses in P3, P12, and P13. The body temperature of both patients increased by more than 39°C after CAR-GPC3 T-cell (CART) infusion and was accompanied by CAR T-cell copy expansion. P3 exhibited rapid elevations in Hs-CRP, IL6, RANTES, and MCP-1 levels, accompanied by the peak CAR-GPC3 DNA copy number of 569 copies/μg genomic DNA on day 14. The patient experienced grade 1 CRS on day 2 and recovered. P13 exhibited rapid elevation of IFNγ, IL10, IL15, and IL6 levels. CRS (grade 2) was diagnosed on day 10 and resolved after tocilizumab and corticosteroid administration. The peak CAR-GPC3 DNA copy number was 23,358 copies/μg genomic DNA on day 15. P12 rapidly exhibited elevation of IL2, IL6, IFNγ and IL10 levels. CRS was diagnosed on day 1 and treated with the tocilizumab and high-dose corticosteroid. The peak CAR-GPC3 DNA copy number was 10,713 copies/μg genomic DNA on day 7. The patient's condition rapidly deteriorated and the patient died on day 19.

CRS is a major clinical concern for patients receiving CAR T-cell therapy (22, 25, 26). Eight subjects experienced low-grade CRS (grade 1 or 2), which was self-limiting and reversible. All patients experienced fever: 11 at grade 1 or 2 and 2 at grade 3. We did not see a clinically meaningful difference in the incidence and severity of CRS when comparing patients who received a dose of Y035 CAR-GPC3 T cells greater than 20.0×10^8 ($n = 2$) with the other patients (data not shown). Four patients received high-dose steroids, two of whom also received the mAb tocilizumab against IL6 receptor to manage CRS (**Table 2**). Surprisingly, one patient experienced grade 5 CRS when given a dose of 20.0×10^8 cells even though 6 patients in the studies had safely received a similar or higher dose of Y035. To the best of our knowledge, this occurrence was the first reported case of grade 5 CRS in a patient with HCC after CAR T-cell therapy.

Patient P12 was a 53-year-old man with metastatic HCC and a GPC3 IHC staining intensity score of 3+. Prior to enrollment, the patient underwent partial hepatectomy, transcatheter arterial chemoembolization, radiofrequency ablation, sorafenib administration, and other targeted HCC therapies. His ECOG performance score was 1, and his body weight was 45 kg. Because of a large tumor burden and high AFP level of 52,096 ng/mL, the patient received microwave ablation therapy for his cancer after screening. However, MRI showed a rapid progression of the disease. About 1 month prior to CAR T-cell therapy, the number of intrahepatic lesions increased from about 10 to more than 30, and the diameter of the largest target lesion increased from 4 to 8 cm. On the day of CAR T-cell infusion, patient P12 had no significant liver function deterioration or progressive weight loss, and the patient's ECOG performance status remained at 1. After the risk of T-cell treatment was described again to the patient and family, the patient was administered Y035 CAR-GPC3 T cells at a dose of 20.0×10^8 cells. The next day, the patient experienced severe CRS-related hypotension, fever, and respiratory difficulties indicating pulmonary edema. The patient was then transferred to the intensive care unit for close monitoring and intensive supportive care, including the administration of tocilizumab and high-dose steroids. Laboratory test results demonstrated an IL6 level greater than 18,000 pg/mL and IL2 level greater than 1,000 pg/mL a few hours after T-cell infusion, and elevation of IFNγ and IL10 levels in the following days (**Fig. 3F**). The patient's condition improved initially after treatment in the intensive care unit. However, the patient P12 died of multiple organ failure and end-stage HCC on day 19.

Antitumor activity of CAR-GPC3 T-cell therapy

We evaluated all 13 patients for antitumor activity. Two patients were alive at the time of data analysis. The survival probabilities at 3 years, 1 year, and 6 months were 10.5%, 42.0%, and 50.3%, respectively, according to the Kaplan–Meier method, with a median OS duration of 278 days (39.7 weeks; 95% confidence interval, 48–615 days; **Fig. 4A** and **B**).

Two patients (P3 and P13) had confirmed PRs (**Fig. 4C**), and one (P1) had a sustainable stable disease (SD). The target lesions in the two patients with PRs exhibited significant shrinkage. Their OS durations were 615 and 385 days, respectively, and their PFS durations were 111 and 99 days, respectively. Patient P13 was alive at the time of data analysis with an AFP level of 1.97 ng/mL (Supplementary Fig. S1B). In addition, patient P1, despite having less than a 30% reduction in the size of target lesions, exhibited a significant clinical benefit of T-cell therapy, with a remarkable reduction in serum AFP level and continued long-term survival. The patient remained alive with a serum AFP level in the normal range at the time of data analysis (**Table 1**; Supplementary Fig. S1B). Of note, this patient had vessel invasion (inferior vena cava tumor thrombus and right atrium tumor thrombus) and metastatic lymph nodes at baseline, which generally indicate a poor prognosis.

We monitored serum AFP levels throughout the studies. Patients P1, P3, and P13, who had clinically meaningful antitumor activity of PD or sustainable SD, had high-percentage reductions in serum AFP levels after Y035 CAR-GPC3 T-cell infusion (Supplementary Fig. S1A).

Discussion

We conducted the first-in-human clinical trials of GPC3-directed CAR T cells in patients with advanced GPC3$^+$ HCC. To our knowledge, this is the first published clinical report of GPC3 CAR-T therapy. Because of a lack of expression in normal tissues and high expression in HCC, GPC3 is considered a promising immunotherapeutic target for HCC. Consistent with a good safety profile observed in a phase I clinical trial of the anti-GPC3 antibody GC33 (35), CAR-GPC3 T cells were tolerated in patients with a good performance status and clinical condition. We expected the most common AEs to be hematologic toxic effects, especially grade 4 lymphocytopenia, due to the preconditioning regimens used prior to CAR T-cell treatment. These toxic effects were recoverable and did not result in any serious infectious AEs. In addition, no grade 3/4 neurotoxic effects or CAR T-cell–related infusion reactions occurred in the studies. The toxic effects potentially associated with the CAR-GPC3 T-cell therapy included CRS and its related symptoms, such as fever and liver function abnormalities. To prevent severe CRS, investigators should make special considerations regarding the eligibility of patients in clinical trials of HCC and pay special attention to the management of AEs in patients with HCC with heavy tumor burdens.

We observed two objective responses to the Y035 CAR-GPC3 T-cell therapy. In addition, one patient with SD also experienced long-term survival (44.2 months). Although a higher GPC3 expression in tumor cells is associated with a worse prognosis for HCC, we did not find a significant correlation between the staining intensity and clinical efficacy in the studies. This correlation will be intensively investigated in the ongoing studies. The antitumor activity of CAR-GPC3 T cells we reported here was more promising than the efficacy reported in the phase I study of mAb GC33. In the GC33 mAb study, the median OS duration was 16.1 weeks (39.7 weeks in our studies), and no patients had objective responses (35). Several advantages of our CAR-T cells may contribute to better clinical outcomes. First, CAR-T cells eliminate the tumor cells by directly lysing the target cells upon engaging the antigen via physiologic cytotoxic lymphocytes and natural killer cells, a mechanism different with mAbs even if they share the same antigen specificity. CAR-T cells are superior to mAbs especially for those target cells with the low expression level of antigen. Second, CAR-T cells have a longer half-life than mAbs. The combination of CAR T cells with local treatment such as thermal ablation may release the tumor antigen and enhance the CAR-T activity. In our studies, CAR DNA copy numbers in the peripheral blood increased rapidly after Y035 CAR-GPC3 T-cell infusion, indicating successful expansion of the T cells. As described previously, the AUC_{last} values for the copy numbers in the two therapy responders tended to be higher than those in the other patients, suggesting a relationship between CAR T-cell persistence and antitumor activity of Y035 CAR-GPC3 T cells. Serum levels of cytokines such as IL6 and IFNγ increased significantly after

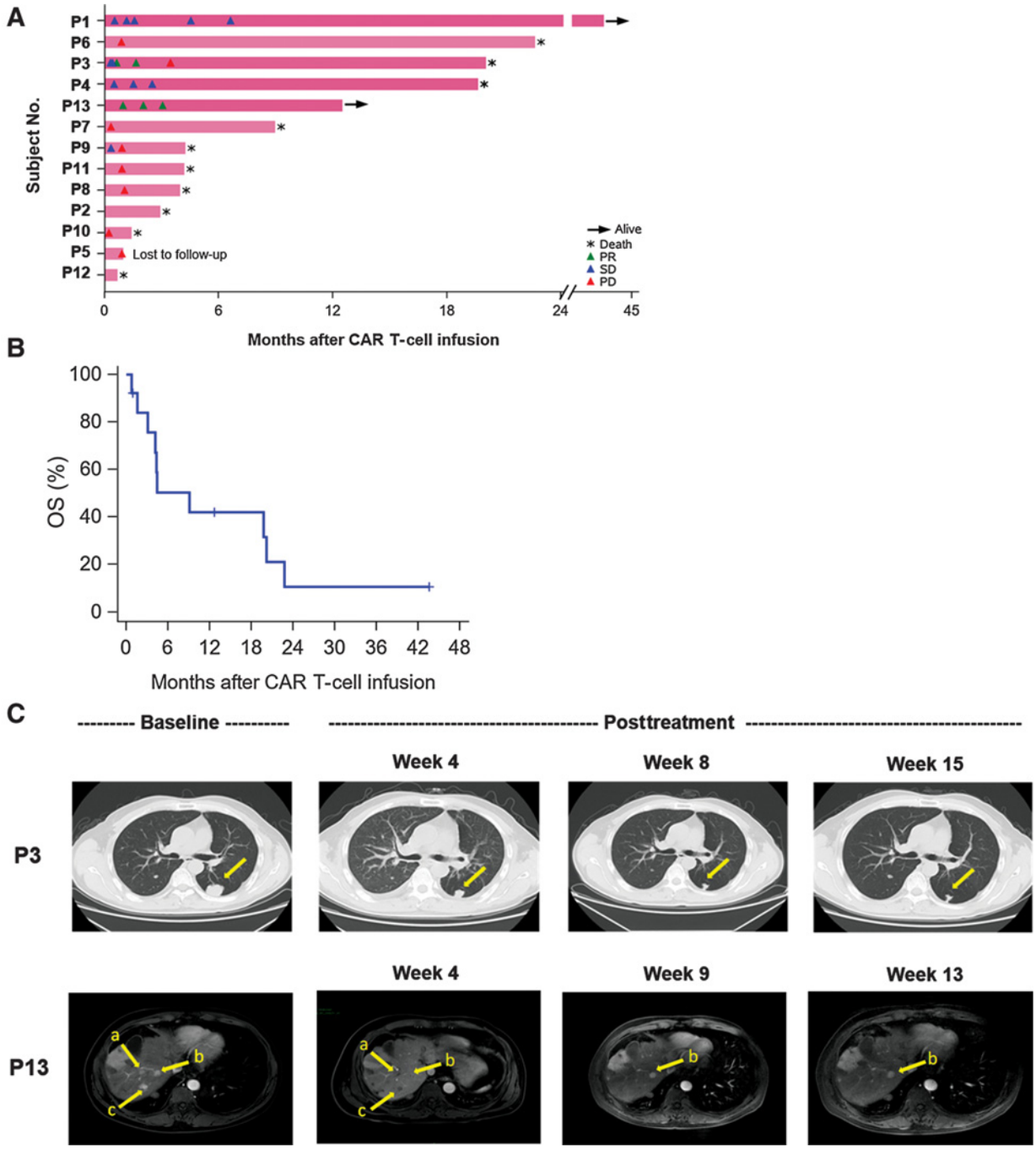


Figure 4.

Clinical responses and exploratory subgroup analysis in patients with HCC administered CAR-GPC3 T cells. **A,** Swimmer's plot of the time after CAR-T for each patient. A total of 13 patients with HCC received CAR-GPC3 T-cell infusions. Eligibility criteria included an expected survival duration of 12 weeks. At the data cutoff point, two patients were still alive. P5 was lost to follow-up after 1 month. PD, progressive disease. **B,** Kaplan–Meier curve of OS for patients. Censored patients (those alive or lost to follow-up at the time of data analysis) are indicated by plus signs. **C,** Tumor responses in two patients with advanced HCC (P3 and P13). CT scans of one target lesion (yellow arrow) in the liver of P3 taken before and after CAR-GPC3 T-cell infusion. The patient was a 50-year man with a multifocal HCC history of 2.2 years and a GPC3 IHC staining intensity score of 3+ prior to enrollment. He experienced grade 1 CRS and grade 2 fever. CT demonstrated a progressive reduction in lesion size from 31.0 × 27.8 mm to 12.2 × 9.5 mm. The patient had a confirmed PR and a PFS duration of 111 days. MRI scans of P13 before and after CAR-GPC3 T-cell treatment. The patient was a 52-year-old male with a multifocal HCC history of 3.7 years. The patient had a GPC3 IHC staining intensity score of 3+. His prior therapies included five transcatheter arterial chemoembolization (TACE) procedures and 11 thermal ablations. He had grade 2 CRS and an IL6 level greater than 1,000 pg/mL on day 15. After receiving multiple doses of tocilizumab, the patient gradually recovered from CRS. MRI scans of multiple lesions in the liver are shown (a, b, and c with yellow arrows). Starting 4 weeks after CAR T-cell infusion, MRI revealed significant reductions in lesion sizes, with lesions a and c disappearing completely and lesion b shrinking slightly from 14.2 × 13.4 mm to 13.9 × 13.0 mm. The patient had confirmed PR and a PFS duration of 99 days. He was alive at the time of data analysis.

CAR T-cell treatment. Such increases may be correlated with clinical symptoms such as elevated body temperature and CRS, confirming the importance of close cytokine level monitoring during CAR T-cell therapy.

The serum AFP level is an important biomarker of tumor response and disease relapse in patients with HCC (42, 43). Normalization of serum AFP levels in patients with high baseline AFP levels is a useful indicator of the success of surgical resection. Furthermore, decreased serum AFP level is associated with improved OS after treatment with sorafenib (44). Indeed, in this study, the three patients with clinically meaningful benefits of the CAR T-cell treatment had greater reductions in serum AFP levels than did the other patients. This finding is consistent with the favorable antitumor activity of the treatment observed in the imaging of these patients.

This report also confirmed that dose modification in patients with HCC with heavy tumor burden is important, particularly when those patients are included in clinical trials of CAR-GPC3 T-cell therapy. To prevent severe CRS, investigators should make special considerations regarding the eligibility of patients in clinical trials of HCC and pay special attention to the management of AEs in patients with HCC with heavy tumor burdens. Optimization of CAR T-cell infusion doses and schedules, as well as pretreatment regimens, is warranted to improve CAR T-cell persistence and tumor penetration. We did not observe any difference in clinical outcomes between patients who received multiple split infusions versus those who received single infusions of CAR-GPC3 T cells. From the point of clinical practice, a single dose of CAR-GPC3 T cells infusion is more likely to be implemented in future clinical trials. Dose-modification strategies such as the reduction of the CAR-GPC3 T-cell dose or a single preconditioning chemotherapeutic agent in patients with HCC with heavy tumor burdens should be explored in future clinical trials. This pilot program of the first ever reported experience with CAR T in HCC will inform future studies to use a unified regimen and dose to validate the signal of activity in HCC. We are currently conducting a CAR-GPC3 clinical trial that is currently the first and only investigational new drug cleared for CAR T research in solid tumors in China.

This report confirms that new CAR T-cell approaches with GPC3 should be considered for HCC. For example, in our previous study of immunocompetent and immunodeficient HCC mouse models (36), we demonstrated the combined antitumor effects of sorafenib and CAR-GPC3 T cells, suggesting that this combination therapy can be applied clinically to HCC. We are investigating whether a different lymphodepletion regimen in the combination of CAR-T cells and a tyrosine kinase inhibitor can be an alternative approach to replace the standard lymphodepletion regimen for patients with HCC, who commonly develop compromised hepatic function. Further improvements of the CAR-GPC3 with next-generation technology are also promising and may not require lymphodepletion pretreatment. For instance, our recent study demonstrated that IL12 armored GPC3-redirected CAR T cells could greatly improve the antitumor activities in mouse model even with large tumor burdens (41). We also showed that introducing an IL4/21 inverted cytokine receptor into the CAR-GPC3 construct improved the CAR T-cell potency *in vitro* and *in vivo* (37). In another recent study, our group disrupted PD-1 gene expression in CAR-GPC3 T cells using the CRISPR/Cas9 gene-editing system. This disruption enhanced the *in vivo* activity of CAR T cells against HCC and improved the persistence and infiltration of CAR T cells in mice bearing HCC (45). These new CAR-GPC3 T-cell therapeutic approaches are promising and should be explored in future HCC clinical trials.

In summary, we report herein the initial safety profile of Y035 CAR-GPC3 T-cell therapy in patients with advanced HCC. Early signs of antitumor activities were observed. Future studies are warranted to further confirm the safety and efficacy of CAR-GPC3 T cells.

Disclosure of Potential Conflicts of Interest

D. Yuan and H. Ma are employees/paid consultants for CARsgen. H. Wang and Z. Li report a patent on the antibody against glypican-3 (CN201610626384.7) issued to CARsgen Therapeutics. No potential conflicts of interest were disclosed by the other authors.

Authors' Contributions

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Writing, review, and/or revision of the manuscript: D. Shi, A.O. Kaseb, H. Ma, Z. Li
Administrative, technical, or material support (ie, reporting or organizing data, constructing databases): Y. Shi, Q. Lu, H. Wang, H. Ma, H. Wang
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