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# Phase I Study of Chimeric Antigen Receptor–Modified T Cells in Patients with EGFR-Positive Advanced Biliary Tract Cancers

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## Abstract

**Purpose:** This study is an expanded and parallel clinical trial of EGFR-specific chimeric antigen receptor–engineered autologous T (CART) cell immunotherapy (NCT01869166) to assess the safety and activity of CART-EGFR cell therapy in EGFR-positive advanced unresectable, relapsed/metastatic biliary tract cancers (BTC).

**Experimental Design:** Patients with EGFR-positive (>50%) advanced unresectable, relapsed/metastatic BTCs were enrolled. Well-produced CART-EGFR cells were infused in a manner of dose escalation after the conditioning treatment with nab-paclitaxel (100–250 mg/m$^2$) and cyclophosphamide (15–35 mg/kg).

**Results:** A total of 19 patients (14 cholangiocarcinomas and 5 gallbladder carcinomas) received one to three cycles of CART-EGFR cell infusion (median CART cell dose, $2.65 \times 10^6$/kg; range, $0.8–4.1 \times 10^6$/kg) within 6 months. The CART-EGFR cell infusion was tolerated, but 3 patients suffered grade ≥3 acute fever/chill. Grade 1/2 target-mediated toxicities including mucosal/cutaneous toxicities and acute pulmonary edema and grade ≥3 lymphopenia and thrombocytopenia related to the conditioning treatment were observed. Of 17 evaluable patients, 1 achieved complete response and 10 achieved stable disease. The median progression-free survival was 4 months (range, 2.5–22 months) from the first cycle of treatment. Analysis of data indicated that the enrichment of central memory T cells (Tcm) in the infused CART-EGFR cells improved the clinical outcome.

**Conclusions:** The CART-EGFR cell immunotherapy was a safe and active strategy for EGFR-positive advanced BTCs. The enrichment of Tcm in the infused CART-EGFR cells could predict clinical response. *Clin Cancer Res; 24(6); 1277–86. ©2017 AACR.*

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## Introduction

Biliary tract cancers (BTC) comprise a group of highly invasive heterogeneous neoplasms including gallbladder carcinoma (GBCA), intrahepatic cholangiocarcinoma (iCCA), perihilar cholangiocarcinoma (pCCA), and distal cholangiocarcinoma (dCCA). Radical resection has been the only possibility for cure; however, approximately 90% of the patients with BTCs lose the possibility of surgical treatment because of the advanced stage of their disease, resulting in a poor prognosis with the median overall survival (OS) rarely exceeding 6 to 8 months (1, 2). For patients diagnosed with unresectable and/or metastatic BTCs, gemcitabine combined with platinum was the recommended standard first-line chemotherapy. Disappointingly, except for 21% to 34.5% of the population with BTCs, most of the patients were insensitive to gemcitabine-based chemotherapy (3–7). The low overall response rates (ORR) and limited survival benefit brought about by chemotherapeutic regimens have driven patients who suffered with advanced BTCs to search for a more effective treatment strategy.

EGFR, a receptor tyrosine kinase, is commonly expressed in BTCs, and an overexpression has been described in nearly all iCCAs, 50% of extrahepatic cholangiocarcinomas (eCCAs), and 38% of GBCAs, indicating that EGFR represents a valid therapeutic target for the treatment of advanced BTCs (8). Unfortunately, the EGFR inhibitor erlotinib as a monotherapy failed to produce promising results in several phase II trials, with the objective response rates of 8% to 12%, and the combination of anti-EGFR antibody with chemotherapy in clinical trials did not show a significant improvement of OS and progression-free survival (PFS) in patients with advanced BTCs (9–14). Recent genetically modified immune cells have illustrated more sensitive and potent antitumor activity than that of a bivalent antibody, even for those with low target antigen expression, which suggested a promising treatment using EGFR-directed genetically modified immune cells in patients with BTCs (15, 16).

Recently, numerous clinical studies of chimeric antigen receptor–modified T (CART) cells have achieved spectacular successes in B-cell hematologic malignancies; meanwhile, CART cell therapy has been actively studied in a number of solid tumors, and these studies have indicated the feasibility and efficacy of CART cell–based immunotherapy in solid malignancies, especially those with certain highly specific tumor-associated antigens (17–23). In our previous study, we demonstrated that

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## Translational Relevance

Biliary tract cancers (BTC) are very common in East Asia, especially in China, although they are rare in Western countries. For patients diagnosed with advanced BTCs, the prognosis is extremely poor due to the unresectable or metastatic lesions and limited efficacy of chemoradiotherapy. Therefore, new treatment regimens to improve the outcome of BTCs are encouraged. In this trial, we designed a CART-EGFR cell immunotherapy conditioned with nab-paclitaxel and cyclophosphamide and studied the potential of this regimen for treating advanced BTCs. Our data showed that CART-EGFR cell therapy was a safe and active strategy in treating EGFR-positive advanced BTCs, providing a new feasible approach that may favor patients with advanced BTCs.

a protocol containing EGFR-specific CART (CART-EGFR) cell infusion was well tolerated, but with grade ≥3 increase of serum lipase in 1 of 11 patients and grade 1/2 EGFR-targeting toxicity, and we also showed an efficient clinical response in patients with EGFR-positive advanced non–small cell lung cancer (NSCLC; ref. 24). In addition, the study illustrated that a proper conditioning chemotherapy administered before CART cell infusion could generate better clinical outcomes when compared with CART cell therapy alone. We realized the importance of tumor stroma and the microenvironment in blocking the antitumor activity of CART cells and therefore tested several chemotherapeutic agents to deplete stroma in our previous report of NSCLC; we also continued to search for an optimal stroma-depleting regimen. It has been reported that albumin-bound paclitaxel (nab-paclitaxel) could be more efficient than other chemotherapeutic drugs in depleting tumor stroma by binding the secreted protein acidic and rich in cysteine (SPARC), which is overexpressed in a variety of malignant tumors including BTCs (25). In this study, we expanded the clinical trial (NCT01869166) by designing a conditioning chemotherapy (nab-paclitaxel and cyclophosphamide)-based CART-EGFR cell therapy to evaluate its safety and efficacy in EGFR-positive advanced unresectable, relapsed/metastatic BTCs.

## Materials and Methods

### Patients

This study (NCT01869166) was approved by the ethics committee of the Chinese PLA General Hospital (Beijing, China). Informed consent was obtained from eligible patients in accordance with the Declaration of Helsinki. No commercial sponsor was involved in the study. Patients were enrolled in the study if they had a diagnosis of advanced unresectable, relapsed/metastatic EGFR-positive BTCs. An expression level of EGFR on tumor cells of more than 50% was a prerequisite for patient enrollment, and EGFR positivity was determined by immunohistochemistry (IHC; ref. 26). Patients were required to have an Eastern Cooperative Oncology Group Performance Status of 0 to 1, have at least one measurable target lesion, have adequate cardiac and pulmonary function, have adequate bone marrow reserve, and have hepatic and renal functions as follows: absolute neutrophil count ≥1,500/mm$^3$, platelet count ≥100,000/mm$^3$, hemoglobin ≥10 g/dL, ALT/AST <2.5 × ULN, total bilirubin <1.5 × ULN, and serum creatinine <1.5 × ULN. Patients were excluded if they had a life expectancy shorter than 3 months. In addition, patients were excluded who had uncontrolled hypertension (>160/100 mmHg), unstable coronary diseases, severe liver and kidney dysfunction, any type of primary immunodeficiency, active virus infections such as hepatitis and human immunodeficiency virus, or pulmonary function abnormalities as follows: forced expiratory volume (FEV) <30% predicted, diffusing capacity of lung for carbon monoxide (DLCO) <30% predicted (following bronchodilator), and oxygen saturation <90% on room air. Patients who were pregnant or lactating or who were participating in any other clinical trials in the prior 30 days were also excluded.

### Study design

This study was designed to assess the safety and efficacy of an adoptive immune cell therapy using autologous T cells expressing an EGFR-specific CAR for the treatment of EGFR-positive advanced unresectable, relapsed/metastatic BTCs. The detailed clinical protocol is described according to the schema in Fig. 1. All patients received contrast-enhanced CT, MRI, and PET/CT to evaluate overall disease burden before conditioning chemotherapy and CART-EGFR cell treatment. Patients received conditioning chemotherapy with nab-paclitaxel at a total dose of 100 to 250 mg/m$^2$, followed by a dose of 15 to 35 mg/kg cyclophosphamide (Table 1). For safety concern, patients received the infusion of CART-EGFR cells with escalating T-cell doses continuously for 3 to 5 days. The detail of dose escalation is provided in the Supplementary Methods. Palliative radiotherapy was administered to relieve tumor-associated pain or other symptoms. Adverse events were documented and graded based on the Common Terminology Criteria for Adverse Events (CTCAE) v4.0. The clinical response was evaluated using CT, MRI, and PET/CT according to the RECIST 1.1 (27). All patients were encouraged to receive three cycles of CART-EGFR cell treatment within 6 months unless there were new tumor lesions or intolerable toxicities. Disease control rate was defined as complete response (CR), partial response (PR), and stable disease (SD) in all evaluable patients. All patients were recruited to receive CART-EGFR cell therapy and follow-up between December 1, 2014, and November 30, 2016.

### Generation of CART-EGFR cells

CART-EGFR cells were generated from patients' autologous peripheral blood mononuclear cells (PBMC) at the Chinese PLA General Hospital Good Manufacturing Practice facility according to a modification of the standard operating procedures. The PBMCs were collected from 80 to 100 mL peripheral blood and stimulated by 50 ng/mL anti-CD3 monoclonal antibody (Takara) and cultured in GT-T551 medium (Takara) with 0.5% autologousserum and 500 U/mL recombinant human IL2 (rhIL2; PeproTech). The transduction was performed after 2 days in cell culture. The lentiviral particles encoding an EGFR-CD137-CD3zeta-CAR was thawed and loaded into 6-well plates coated with 10 μg/mL RetroNectin (Takara) at 4°C overnight. Virus-loaded plates were centrifuged at 2,000 *g* for 2 hours at 32°C. Subsequently, 5 × 10$^5$ activated cells were added to prewells, followed by centrifugation at 1,000 *g* for 10 minutes. After overnight culture, the cells were placed in fresh GT-T551 medium with 0.5% autologous serum and 500 U/ml rhIL2 and further expanded in culture bags (Takara). Cell infusion was performed after a total of 10 days in culture. Release criteria included negative bacterial, fungal, and *Mycoplasma* cultures after transduction and on the day of infusion; endotoxin

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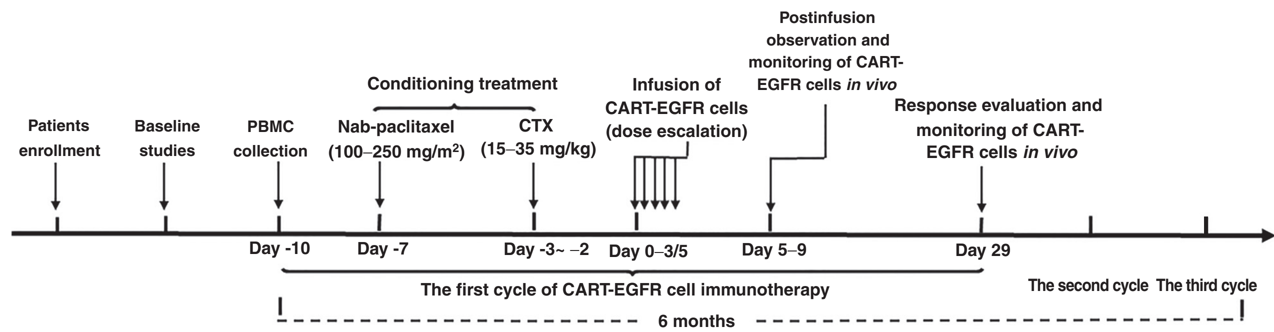


**Figure 1.**
Schema of clinical protocol. CTX, cyclophosphamide; PBMC, peripheral blood mononuclear cell.

level ≤5 EU/kg after transduction and on the day of infusion; Gram stain negative on the day of infusion; ≥80% cell viability, immunophenotype, and detection of CAR expression (>5%) by flow cytometry; and EGFR-specificity cytotoxicity.

### Statistical analysis

GraphPad Prism version 5.0 for Windows was used to perform the statistical analysis. The outcomes are shown as mean ± standard deviations, and the number of infused CART-EGFR cells

**Table 1.** Patients' clinical characteristics

| Patient number | Sex | Age (years) | Diagnosis | Status at enrollment | Cycles of CART cell therapy | Conditioning regimens nab-P (mg/m$^2$), CTX (mg/kg) | CAR-positive T cells in each cycle (× $10^6$/kg) | Best response | PFS (months) |
|---|---|---|---|---|---|---|---|---|---|
| 1 | Female | 52 | dCCA | Unresectable | 1 | nab-P 241.0. CTX 28.1 | 4.1 | CR | 22[a] |
| 2 | Female | 53 | GBCA | Unresectable | 2 | nab-P 191.1. CTX 22.2 (1st)<br>nab-P 191.1. CTX 22.2 (2nd) | 3.5 (1st)<br>2.8 (2nd) | SD | 4 |
| 3 | Male | 70 | pCCA | Relapsed/metastatic | 3 | nab-P 112.4. CTX 22.9 (1st)<br>nab-P 168.5. CTX 23.5 (2nd)<br>nab-P 170.5. CTX 22.5 (3rd) | 2.5 (1st)<br>2.1 (2nd)<br>2.0 (3rd) | SD | 9.5 |
| 4 | Male | 68 | GBCA | Relapsed/metastatic | 1 | nab-P 161.3.[b] | 1.5 | SD | 15 |
| 5 | Male | 59 | iCCA | Relapsed/metastatic | 1 | nab-P 160.4. CTX 21.3 | 1.1 | PD | |
| 6 | Female | 63 | GBCA | Unresectable | 1 | nab-P 188.7. CTX 23.3 | 3.4 | PD | |
| 7 | Male | 65 | iCCA | Relapsed/metastatic | 1 | nab-P 164.8. CTX 21.1 | 2.0 | Loss of follow-up | |
| 8 | Male | 54 | iCCA | Relapsed/metastatic | 2 | nab-P 177.5. CTX 25.0 (1st)<br>nab-P 180.7. CTX 23.0 (2nd) | 2.8 (1st)<br>3.3 (2nd) | PD | |
| 9 | Male | 39 | dCCA | Unresectable | 3 | nab-P 176.5. CTX 19.4 (1st)<br>nab-P 115.6. CTX 18.5 (2nd)<br>nab-P 125.6. CTX 19.5 (3rd) | 2.6 (1st)<br>2.7 (2nd)<br>2.3 (3rd) | SD | 8 |
| 10 | Female | 64 | pCCA | Relapsed/metastatic | 1 | nab-P 143.9. CTX 12.5[c] | 3.2 | SD | 2.5 |
| 11 | Female | 58 | GBCA | Relapsed/metastatic | 1 | nab-P 181.8. CTX 25.8 | 2.2 | PD | |
| 12 | Female | 45 | GBCA | Relapsed/metastatic | 2 | nab-P 184.0. CTX 26.7 (1st)<br>nab-P 123.5. CTX 20.3 (2nd) | 2.9 (1st)<br>2.8 (2nd) | PD | |
| 13 | Male | 52 | dCCA | Relapsed/metastatic | 3 | nab-P 180.7. CTX 33.3 (1st)<br>nab-P 181.2. CTX 34.3 (2nd)<br>nab-P 181.6. CTX 34.8 (3rd) | 3.1 (1st)<br>2.7 (2nd)<br>1.6 (3rd) | SD | 7.5 |
| 14 | Male | 67 | iCCA | Unresectable | 2 | nab-P 169.5. CTX 29.4 (1st)<br>nab-P 173.4. CTX 30.8 (2nd) | 3.5 (1st)<br>1.7 2nd) | SD | 3 |
| 15 | Male | 45 | iCCA | Relapsed/metastatic | 2 | nab-P 163.0. CTX 27.8 (1st)<br>nab-P 161.3. CTX 21.9 (2nd) | 0.8 (1st)<br>1.4 (2nd) | SD | 4 |
| 16 | Female | 54 | iCCA | Relapsed/metastatic | 2 | nab-P 185.2. CTX 26.2 (1st)<br>nab-P 191.1. CTX 27.8 (2nd) | 1.5 (1st)<br>3.4 (2nd) | SD | 2.5 |
| 17 | Female | 57 | dCCA | Relapsed/metastatic | 1 | nab-P 158.7. CTX 31.7 | 2.8 | Loss of follow-up | |
| 18 | Male | 60 | dCCA | Relapsed/metastatic | 2 | nab-P 179.6. CTX 25.6 (1st)<br>nab-P 180.7. CTX 12.9 (2nd)c | 1.2 (1st)<br>2.5 (2nd) | SD | 4 |
| 19 | Female | 56 | iCCA | Unresectable | 1 | nab-P 185.2. CTX 26.2 | 3.2 | PD | |

Abbreviations: CTX, cyclophosphamide; nab-P, nab-paclitaxel; PD, progression of disease.
[a]Ongoing response.
[b]CTX was canceled for a grade 3/4 decrease of platelet.
[c]CTX was reduced for a grade 1/2 decrease of platelet.

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**Table 2.** Adverse events related to CART-EGFR cell therapy

| | Conditioning | | Infusion | | Follow-up | |
|---|---|---|---|---|---|---|
| | Grade 1/2 | Grade 3/4 | Grade 1/2 | Grade 3/4 | Grade 1/2 | Grade 3/4 |
| **Adverse events** | ***N* (%)** | ***N* (%)** | ***N* (%)** | ***N* (%)** | ***N* (%)** | ***N* (%** |
| Mucosal/cutaneous | | | | | | |
| Oral mucositis | | | 2 (10.5) | | | |
| Oral ulcer | 1 (5.3) | | 5 (26.3) | | | |
| Gastrointestinal hemorrhage | | | | | 1 (5.3) | |
| Desquamation | | | | | 5 (26.3) | |
| Pruritus | | | | | 2 (10.5) | |
| Hematologic | | | | | | |
| Anemia | 8 (42.1) | | 16 (84.2) | | 2 (10.5) | |
| Lymphopenia | 7 (36.8) | 10 (52.6) | 2 (10.5) | 16 (84.2) | 1 (5.3) | |
| Thrombocytopenia | 6 (31.6) | 2 (10.5) | 5 (26.3) | 1 (5.3) | | |
| Others | | | | | | |
| Acute fever/chill | 6 (31.6) | | 16 (84.2) | 3 (15.8) | | |
| Delayed fever/chill | | | | | 7 (36.9) | |
| Fatigue | 15 (78.9) | | 16 (84.2) | | 16 (84.2) | |
| Ascites/pleural effusion | | | 4 (21.1) | | | |
| Acute pulmonary edema | | | 1 (5.3) | | | |
| Diarrhea | | | 2 (10.5) | | 1 (5.3) | |
| Nausea/vomiting | 13 (68.4) | | 3 (15.8) | | | |
| Myalgia/arthralgia | 8 (42.1) | | 2 (10.5) | | | |

was shown as median. Descriptive statistics were used to summarize the data in multiplex analyses. PFS was determined by the Kaplan–Meier method. The relationships between CAR transgene copies and the dose of infused CART-EGFR cells, transgene copies and the number of central memory T cells (Tcm) in infused CART-EGFR cell products, as well as transgene copies and lymphocyte counts were analyzed by linear regression models. The significance of difference between groups was determined by using a *t* test. A *P* value less than 0.05 was considered statistically significant.

## Results

### Patient characteristics

Nineteen eligible patients received the CART-EGFR cell treatment (Supplementary Fig. S1), and the clinical characteristics of these 19 patients are listed in Table 1. Their median age was 57 years (range, 39–70 years), and 53% were males. The 19 patients included 14 with cholangiocarcinoma and 5 with GBCA. The EGFR expression was determined by IHC and shown in Supplementary Table S1. The disease status of the 19 patients was unresectable or relapsed/metastatic BTC before the administration of this regimen (6 had unresectable BTCs and 13 had relapsed/metastatic BTCs).

### Generation and characteristics of CART-EGFR cells

The CART-EGFR cells were successfully generated for all patients. After a 10-day culture period, CART-EGFR cell products were harvested for infusion. Of the infused cells, a mean of 96.5% were $CD3^+$ T cells, with a mean CD4:CD8 percentage of 30.1:68.2 (Supplementary Fig. S2A). Furthermore, a mean of 29.1% of infused cells were Tcm ($CD3^+CD45RO^+CD62L^+CCR7^+$), and representative data are shown in Supplementary Fig. S2B. In addition, 8.6% (mean range, 6.3%–11.2%) of the infused cells were CAR-EGFR positive (Supplementary Fig. S2C). In a CCK-8 detection assay, CART-EGFR cells could significantly lyse EGFR-positive tumor cells (A549 and MCF7), whereas T cells with no transduction or mock transduction could not ($P < 0.05$; Supplementary Fig. S2D).

### Administration of CART-EGFR cells

Nineteen patients received the CART-EGFR cell treatment within 6 months (Supplementary Fig. S1). Patients received a total of 32 cycles of this regimen, including 7 patients who received two cycles and 3 who received three cycles. After conditioning chemotherapy, patients received $2.65 \times 10^6$/kg (median range, 0.8–$4.1 \times 10^6$/kg) CART-EGFR cells (Table 1).

### Toxicities

The adverse events of the 19 patients who received the CART-EGFR cell treatment were summarized and categorized according to CTCAE v4.0 (Table 2). All the toxicities could be reversed, and no treatment-related deaths occurred in this study. Occurrences of grade $\geq$3 lymphopenia (10 of 19) were observed because of lymphodepletion resulting from prior conditioning chemotherapy, and most patients recovered to the normal level within the first week from the cell infusion (Supplementary Fig. S3A). In addition, grade$\geq$3 thrombocytopenia occurred in 2 of 19 patients, and this was thought to be related to conditioning chemotherapy. Anemia influenced by conditioning chemotherapy was also observed in some patients. The CART-EGFR cell infusion was tolerated, with grade $\geq$3 acute fever/chill occurring in 3 of 19 patients. It is important to note that grade 1/2 oral mucositis, oral ulcer, gastrointestinal hemorrhage, desquamation, and pruritus occurred in several patients after the cell infusion. In addition, patient 4 developed acute respiratory distress syndrome during the cell-infusion period. The subsequent CT scan showed that acute pulmonary edema occurred in both lungs, and serum measurements indicated a rapid elevation of IL6 (1,712 pg/mL) and C-reactive protein (CRP; 32.1 mg/dL) compared with baseline. Tocilizumab was administered immediately, leading to the recovery of the patient with acute pulmonary edema after 25 days (Fig. 2A and B). Concentrations of all the patients' serum cytokines including IL2, IL6, IL8, and TNFα as well as CRP

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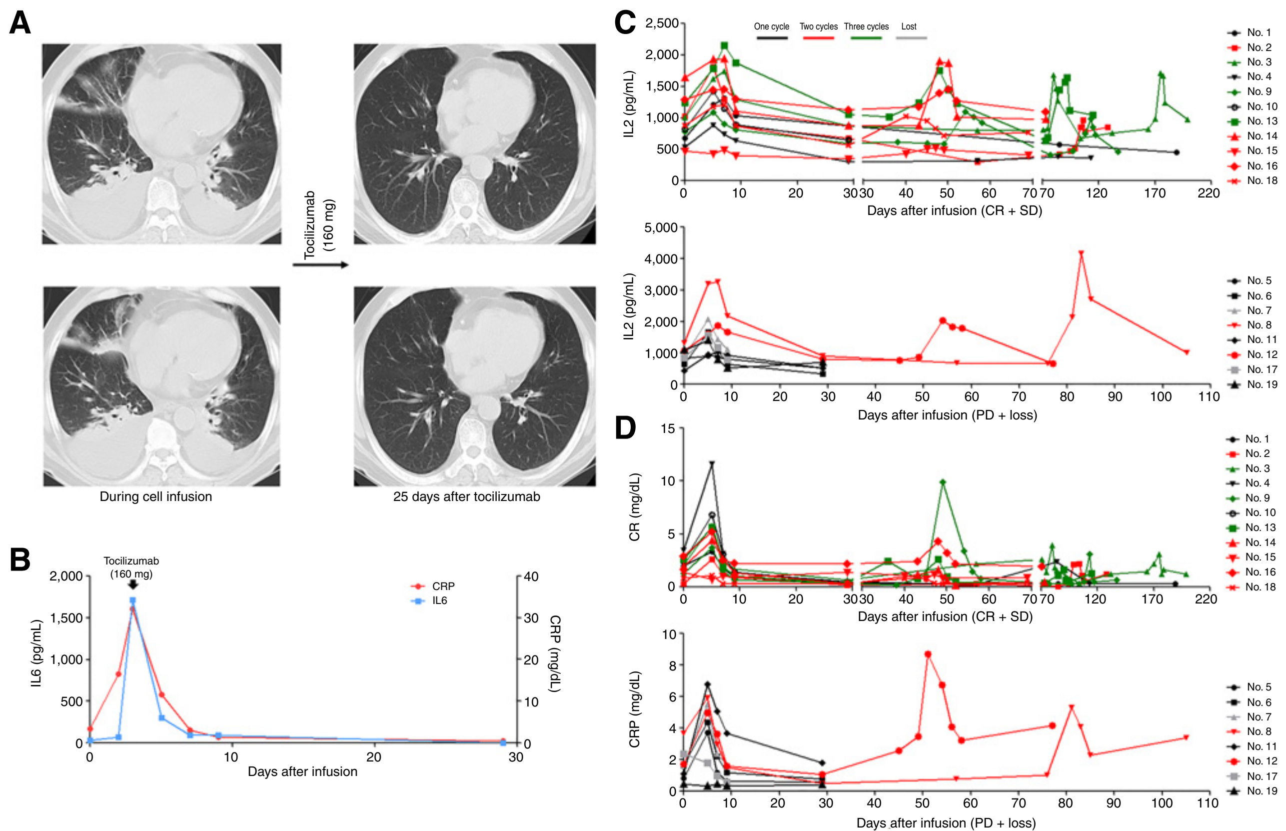


**Figure 2.**

Changes in all patients' serum IL2 and CRP levels and lung toxicity in patient 4. **A,** CT scan showed acute pulmonary edema in patient 4 during the CART-EGFR cell-infusion period and indicated that acute pulmonary edema recovered 25 days after the administration of tocilizumab. **B,** Rapid elevation of IL6 and CRP in patient 4 during the CART-EGFR cell-infusion period, and recovery after tocilizumab administration. **C** and **D,** Levels of serum IL2 and CRP were tested in patients before and at serial time points after CART-EGFR cell infusion, and there was a significant increase after CART-EGFR cell infusion ($P < 0.05$). Top (**C** and **D**), the level of IL2 and CRP in patients who achieved CR or SD; bottom (**C** and **D**), the level of IL2 and CRP in patients with progression of disease (PD) or loss of follow-up. Black lines, patients received one cycle of treatment; red lines, two cycles of treatments; green lines, three cycles of treatments; and gray lines, patients who experienced loss of follow-up after receiving the first cycle of treatment. The first cycle of cell infusion was administered at day 0; second was at day 40 (Nos. 15 and 18), day 43 (Nos. 13, 14, and 16), day 49 (No. 9 and 12), day 75 (No. 3), day 76 (No. 8), and day 99 (No. 2); and third was at day 85 (No. 13), day 108 (No. 9), and day 170 (No. 3). No., number.

were determined at serial time points before and after CART-EGFR cell infusion. Levels of IL2 and CRP significantly increased after CART-EGFR cell infusion ($P < 0.05$) and recovered to baseline within the first week from the cell infusion (Fig. 2C and D, Supplementary Fig. S4, and Supplementary Table S2).

## Clinical response

Two of the 19 enrolled patients lost follow-up after the first cycle of CART-EGFR cell treatment. Of the 17 evaluable patients, 1 achieved CR for 22 months and 10 had SD for 2.5 months to 15 months from the first cycle of treatment (Fig. 3A and B), and the detailed data are listed in Table 1. The clinical response assessment of the 17 evaluable patients was observed for at least 1 month. The median PFS was 4 months (range, 2.5–22 months; Fig. 3C), and 1 patient continued to have a response at the time of writing. The CT and PET/CT scans showed that patient 1, who had advanced unresectable dCCA with retroperitoneal lymph node metastasis, achieved a CR at 3 months after receiving this treatment, including the disappearance of the abnormal intense metabolic primary tumor lesion and metastatic retroperitoneal lymph node (Fig. 3D; Supplementary Fig. S5). The PFS of this patient lasted for more than 22 months and is ongoing at the time of writing (Fig. 3B).

## Relationship between *in vivo* persistence of CART-EGFR cells and clinical response

The persistence of CART-EGFR cells was assessed using quantitative real-time PCR (qRT-PCR) to detect the CAR gene copy number in patients' peripheral blood (Fig. 4A; Supplementary Table S2). There was a rapid increase of CAR gene copies after CART-EGFR cell infusion. The copy numbers gradually declined close to baseline within 1 month, but a low-level signal could be detected 21 months after treatment in patient 1, who had only received one cycle of treatment. Linear regression analysis indicated no significant correlation between gene copies after CART-EGFR cell infusion and the dose of infused CART-EGFR cells (Supplementary Fig. S6A), and there were no significant differences between the dose of infused CART-EGFR cells in patients who achieved different

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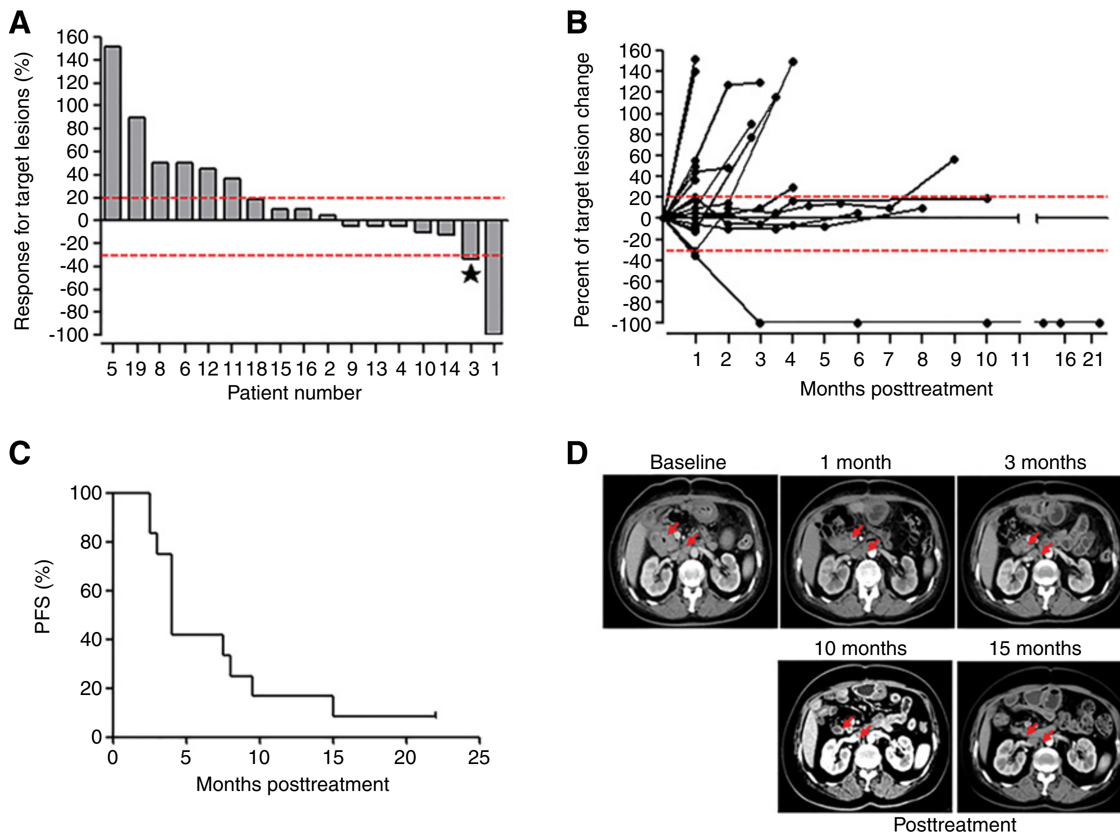


**Figure 3.**

Clinical outcome after the CART-EGFR cell treatment. **A** and **B,** Clinical outcome from baseline in the sum of the longest diameters of target lesions as assessed by RECIST 1.1 in patients who had more than one postbaseline tumor assessment. Zero indicates baseline; the range between two red lines indicates SD. **A,** The best patient clinical response following this treatment. ★ indicates that patient 3 was assessed as having a PR 1 month after this treatment, which was modified to SD when reassessed 1 month later. **B,** Percentage change from baseline following the CART-EGFR treatment. **C,** PFS of all evaluable patients was analyzed by the Kaplan–Meier method. The median PFS was 4 months. **D,** CT scan images of patient 1 before (baseline) and 1, 3, 10, and 15 months after the CART-RGFR cell treatment. Red arrows indicate primary tumor and retroperitoneal lymph node metastasis.

clinical responses (Supplementary Fig. S6B). Gene copies at day 29 after CART-EGFR cell infusion in patients who achieved CR/SD were statistically significantly higher than in progression of disease (PD) patients ($P = 0.0465$; Fig. 4B; Supplementary Fig. S6C).

The persistence of CART cells in patients has been found to be highly concordant with the percentage of Tcm in infused CART cell products (28). Herein, we analyzed the correlation between patients' CAR transgene copies and the number of Tcm in CART-EGFR cell products, and we found that there was a significant correlation between them ($P = 0.0022$; Fig. 4C). In addition, the number of Tcm in the infused cell products in patients who achieved CR/SD was significantly higher than that in PD patients by using the $t$ test analysis ($P = 0.0464$; Fig. 4D).

## Discussion

In this phase I study, we established the safety and efficacy of the CART-EGFR cell therapy in 17 evaluable patients with EGFR-positive advanced unresectable, relapsed/metastatic BTCs. One patient achieved CR, and 10 had SD. The median PFS was 4 months from the first cycle of treatment. Statistical analysis indicated that the enrichment of Tcm in CART-EGFR cells could improve the clinical outcome.

On-target/off-tumor toxicity is still a major concern regarding CART cell therapy for solid tumor treatment; this toxicity is caused when CART cells injure healthy cells. In the original study on NSCLC treated with CART-EGFR cells, tolerable and controlled EGFR-related targeting toxicities indicated that therapy with anti-EGFR CAR was an appropriate treatment option (24). Overall, infusion of CART-EGFR cells up to $4.1 \times 10^6$/kg was tolerated in this study (Table 2). Hematologic adverse events including anemia, lymphopenia, and thrombocytopenia had a high correlation with prior conditioning chemotherapy. The most likely on-target/off-tumor adverse events related to CART-EGFR cells were mucosal/cutaneous toxicities including oral mucositis, oral ulcer, gastrointestinal hemorrhage, desquamation, and pruritus. In addition, patient 4 developed acute respiratory distress syndrome during the cell-infusion period and displayed an acute pulmonary edema accompanying a rapid elevation of serum IL6 and CRP. The patient's serum IL6 and CRP level and acute pulmonary edema recovered after tocilizumab (an anti-IL6 antibody) administration, which has been reported to control cytokine release syndrome to an acceptable degree (29). The data indicated the

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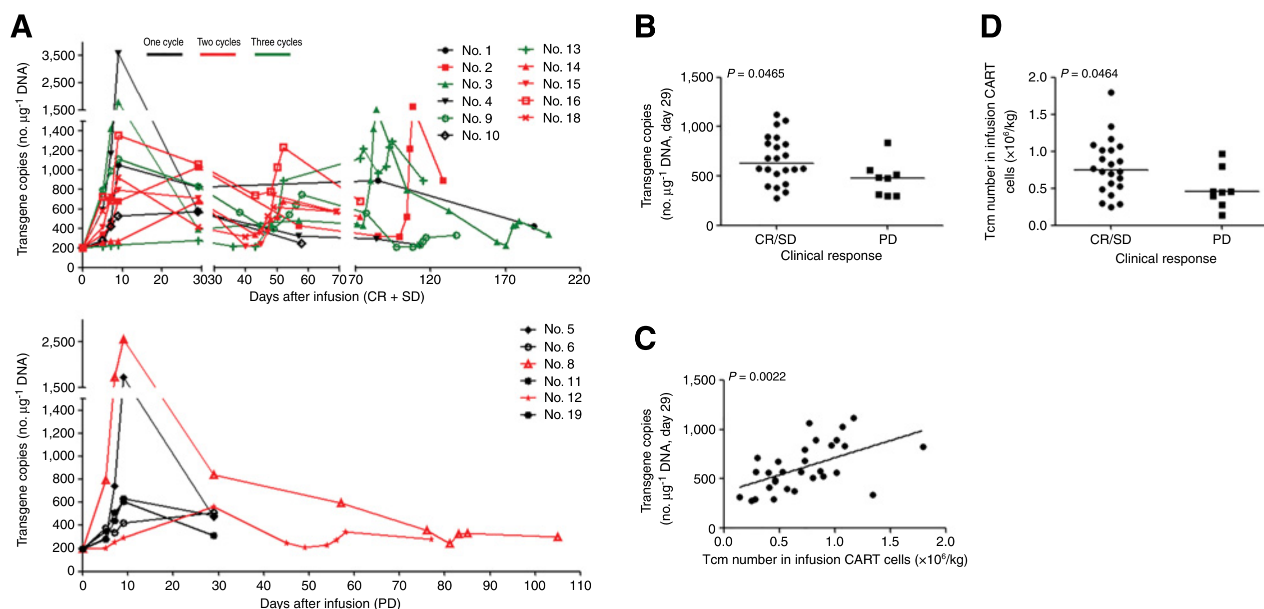


**Figure 4.**

Persistence of CART-EGFR cells in patients with EGFR-positive advanced biliary tract cancers. **A,** Persistence of infused CART-EGFR cells in patients' peripheral blood. The level of CART-EGFR cells was assessed using qRT-PCR to detect the CAR gene copy number in genomic DNA obtained from patients' peripheral blood, the samples of which were divided into two groups based on clinical response. Top, the persistence of CART-EGFR cells in patients who achieved CR or SD; bottom, persistence in patients with PD. Black lines, patients received one cycle of treatment; red lines, two cycles of treatments; and green lines, three cycles of treatments. **B,** Comparison of CAR transgene copies at 29 days after CART-EGFR cell infusion in patients who achieved different clinical responses. The *t* test was used to analyze the difference between the two groups, CR/SD and PD. **C,** Using linear regression analysis, it was determined that the CAR transgene copies at day 29 after CART-EGFR cell infusion were correlated with the number of Tcm in infused CART-EGFR cell products. **D,** The *t* test analysis indicated that there was a significant difference for Tcm number in infused CART-EGFR cell products between the two groups of patients who achieved CR/SD and PD following the treatment.

appearance of on-target/off-tumor toxicity and indirectly confirmed effective antitumor activity of the CART-EGFR cells because EGFR is expressed on both human epithelial cancers and most epithelial cells (24, 30). To avoid on-target/off-tumor toxicities, more research is needed to establish factors such as cell dose; an appropriate target; and the optimization of CAR that includes the incorporation of suicide genes, cytotoxic T lymphocyte antigen-4, or programmed death-1 (PD-1)–based inhibitory CAR and the tandem CAR (31–34). In future studies, these safety strategies will enable more extensive use of CART cells in solid tumor treatment.

Prior conditioning chemotherapy has been reported to improve the antitumor effect of CART cells by several potential mechanisms, such as depleting leukocytes, improving the susceptibility of tumor cells to T-cell lysis, increasing the number of CART cells, and debulking the tumor (19, 35, 36). Recent studies showed that cyclophosphamide could be a conditioning agent in the treatment of liquid tumors by causing significant lymphopenia (19, 37, 38). In contrast to hematologic malignancies, solid tumors have a more complex tumor microenvironment and heterogeneity. However, we believe that proper conditioning could improve the clinical outcome of CART cell therapy in solid tumor treatment. The optimal regimen and dose of conditioning chemotherapy are not well-known in solid tumor treatment with CART cells. Nab-paclitaxel has been reported to damage the tumor stroma through binding SPARC, which is overexpressed on BTCs. In this study, cyclophosphamide and nab-paclitaxel were used as conditioning chemotherapeutic agents administered prior to CART-EGFR cell infusion. After conditioning treatment, 17 patients experienced lymphopenia (Table 2), possibly providing an appropriate "lymphoid space" to accommodate CART cells and improve their expansion. The analysis of correlation between lymphocyte counts and clinical response revealed no significant difference of lymphocyte counts between the two groups of patients who achieved CR/SD and PD (Supplementary Fig. S3B). In addition, the data analysis indicated that clinical response was correlated with the percentage of Tcm in infused CART-EGFR cell products (Fig. 4D). Here, it is hard to draw a conclusion that the clinical response may not be directly correlated with lymphodepletion by cyclophosphamide due to the complex tumor microenvironment in patients with solid tumors. Our original study on NSCLC indicated that the administration of conditioning chemotherapeutic agents to damage the tumor stroma could increase clinical benefit (24). However, because only 1 patient achieved a CR after receiving the treatment in this study, we speculate that this chemotherapy regimen is possibly insufficient to damage the tumor stroma. Unfortunately, this concept could not be tested by immunohistochemical techniques because there was no biopsy tissue of the tumor mass from these patients. Therefore, the optimal regimen and dose of prior conditioning chemotherapy in solid tumor treatment remains to be explored further to enhance antitumor activity of CART cells.

Three special patients (SP) with advanced BTCs received compassionate therapy with only CART-EGFR cell infusion, and the details are summarized in Supplementary Special Presentations.

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After cell infusion, SP 1 achieved a PR, SP 2 achieved a CR, and SP 3 had SD. The persistence of CART-EGFR cells in the SPs is shown in Supplementary Fig. S7B and seemed to be higher than or similar to that of the 17 enrolled patients who received the CART-EGFR cell therapy with conditioning chemotherapy. The mechanism to explain the clinical outcome and the *in vivo* persistence of CART-EGFR cells probably included the correlation noted above, which showed that clinical response and CART-EGFR cell persistence were highly associated with Tcm number in infused CART-EGFR cells, not with lymphodepletion. The Tcm number in the first cycle of infused CART-EGFR cells administered to the SPs was higher than the mean of this number in patients who had PD after receiving the CART-EGFR cell therapy with conditioning chemotherapy (Supplementary Fig. S7A). Some particular patient features were of note: SP 1 received radiotherapy 1 month before CART-EGFR cell infusion, which could damage tumor stroma and enhance the antitumor activity of CART cells (39, 40); for SP 2, there was no need to damage the tumor stroma because this patient showed only malignant pleural effusion; and SP 3 had multiple nonmeasurable lesions, which suggested the possibility of having thin tumor stroma (41). These may be the major factors that led the patients to have better or similar clinical outcomes compared with the 17 enrolled patients. In addition, the release of tumor-associated antigen caused by radiotherapy may lead to the rapid expansion of CART cells in response to released antigen in the peripheral circulation (39, 40, 42). Taking all these points into account, we emphasize the importance of damaging tumor stroma or selecting thin tumor stroma for improving the antitumor efficacy of CART cells.

Recent studies have indicated that the persistence of CART cells is associated with improved outcome, which could be correlated with the percentage of Tcm in CART cells (28, 43). Our current data show the correlation of CART-EGFR cell persistence with the number of Tcm in CART-EGFR cell products. Importantly, our data suggest that the enrichment of this population in CART-EGFR cell products is associated with improved clinical outcome. Interestingly, less differentiated T-cell subsets, such as T-memory stem cells that expressed CD45RA, CD62L, and CCR7, have been reported to show higher proliferation, survival, and antitumor activity in mouse xenograft models than even Tcm (44, 45). To modify and optimize the CART cell culture procedure to enrich T-memory stem cells or other less differentiated T cells probably could produce better clinical outcome and would have a crucial impact on the future of CART cell therapy in solid tumors; however, the clinical application of CART cells that contain enrichment of these less differentiated T-cell subsets or Tcm in patients with cancer is still a matter that needs investigation and remains to be confirmed.

This and our original study demonstrated that CART-EGFR cells could mediate potent *in vitro* cytolysis; however, the antitumor efficacy of these cells exerted in patients is less encouraging. The reasons for that are multiple, in which T-cell exhaustion is reported to be a significant barrier that limits the antitumor responses of engineered T cells in the setting of chronic antigen exposure, which could upregulate the expression of inhibitory receptors and their ligands in tumor cells such as programmed death-1 ligands (PD-L1; refs. 46, 47). Therefore, refueling the exhausted CART cells via the PD-1/PD-L1 pathway using anti–PD-1 therapy is considered to be an important approach with potential benefits (48). Several studies have indicated that PD-1/PD-L1 blockade could improve the antitumor efficacy of CART cells in solid tumors (49, 50). In one case report, a patient with refractory diffuse large B-cell lymphoma and progressive lymphoma after therapy with CART-CD19 cells was administered a PD-1–blocking antibody and had a significant antitumor response and an expansion of CART cells (51). However, the aggravation of on-target/off-tumor toxicity may be cocurrent with the enhanced antitumor potency of CART cells by the combination with immune checkpoint blockade.

In conclusion, we established the safety and efficacy of treating patients with advanced unresectable, relapsed/metastatic BTCs with CART-EGFR cell therapy. Based on our findings, further clinical studies for optimizing conditioning chemotherapy are needed to enhance the efficacy of CART cells in solid tumors. In addition, although many obstacles have limited the application of CART therapy in the fight against solid tumors and more work is needed to increase the clinical response in patients with solid tumors, the overall future of cancer immunotherapy is very encouraging.

## Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

## Authors' Contributions

**Conception and design:** Y. Guo, K. Feng, W. Han
**Development of methodology:** Y. Guo, K. Feng, H. Dai, Y. Wang, W. Han
**Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.):** Y. Guo, K. Feng, Y. Liu, Q. Yang, H. Jia
**Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis):** Y. Guo, K. Feng, Y. Liu, Q. Yang, H. Jia, W. Han
**Writing, review, and/or revision of the manuscript:** Y. Guo, K. Feng
**Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases):** Y. Guo, Z. Wu
**Study supervision:** Y. Guo, Y. Liu, H. Dai, W. Han

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