World Journal of Surgery and Surgical Research

Research Article
Published: 18 Mar, 2022

Safety and Efficacy Evaluation of MSLN-Chimeric Antigen Receptor T Cells Secreting Anti-PD-1 Antibodies in the Treatment of Advanced Metastatic Cancers

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Received Date: *28 Jan 2022*
Accepted Date: *03 Mar 2022*
Published Date: *18 Mar 2022*

Citation:
Xia Y, Zhang F, Wang P, Guan X, Wang X, Lai S, et al. Safety and Efficacy Evaluation of MSLN-Chimeric Antigen Receptor T Cells Secreting Anti-PD-1 Antibodies in the Treatment of Advanced Metastatic Cancers. World J Surg Surgical Res. 2022; 5: 1369.

Abstract

Objectives: Although Chimeric Antigen Receptor T (CAR-T) cells exhibit a potent therapeutic effect in B-lineage hematologic malignancies, CAR-T therapy for the heterogeneous solid tumors remains challenging. Tumor immunosuppressive micro-environment within the solid tumor may inhibit the activities of the CAR-T cells. To overcome this obstacle, we generated -MSLN-CAR-T cells secreting anti-Programmed cell Death-1 (PD-1) antibodies (αPD-1-MSLN-CAR-T cells) based piggyBac transposon system and performed a pilot study to assess the safety and efficacy of αPD-1-MSLN-CAR-T cells in the treatment of advanced metastatic malignancies.

Methods: An exploratory multicenter clinical trial was conducted from July 2018 to July 2019. Advanced patients over-expressed with Mesothelin (MSLN) and Programmed Death-Ligand 1 (PD-L1) received one cycles of αPD-1-MSLN-CAR-T cells with a cell dose of 2×10^6 cells/kg. Nine patients were recruited in this study who met the requirements of the clinical trial. Safety and progression-free survival were the primary endpoints and overall survival was the secondary endpoint.

Results: Amongst the 9 cases that participated in the study, 2 cases exhibited a partial remission, and 4 cases achieved stable disease after cell transfusion. The median progression-free survival and overall survival were 4.2 months (95% CI, 2.7–5.7 months) and 6.5 months (95% CI, 4.7–8.3 months), respectively. One case developed fever and Cytokine Release Syndrome (CRS) (grade 1). Most cases had grade 3/4 decreases in lymphocyte counts.

Conclusion: Our data primarily indicate that αPD-1-MSLN- CAR-T cell therapy exhibits a demonstrated effect in advanced metastatic malignancies with low toxicities.

Keywords: Mesothelin; Chimeric antigen receptor; Immunotherapy; PD-1; Malignancy

Introduction

Chimeric Antigen Receptor-T (CAR-T) cell therapy is a novel cancer treatment that has been used in a variety of tumors. CAR-T cells can selectively and directly recognize and kill tumor cells without the requirement of antigen presentation, which is not restricted by the Major Histocompatibility Complex (MHC) [1]. CD19-targeted CAR-T cell therapy has revolutionized the treatment of B cell lymphoma, Acute Lymphoblastic Leukemia (ALL), and refractory multiple myeloma [2-5]. Several cell surface tumor-associated proteins have been used as CAR-T cell targets, including Mesothelin (MSLN), Glypican-3 (GPC3), Prostate-Specific Membrane Antigen (PSMA) and Interleukin-13 Receptor Alpha 2 (IL13Rα2) [6-10].

Mesothelin is a glycoprotein that is anchored to the plasma membrane by a Glycosylphosphatidylinositol (GPI) domain. Generally, mesothelin is expressed in normal mesothelial cells of the pericardium, the pleura, and peritoneum and also in epithelial cells of the

ovaries, tunica vaginalis, rete testis, and fallopian tubes. Mesothelin is highly expressed in nearly all cases of pleura mesothelioma and 82% of serous epithelial ovarian cancers [11-13]. High expression levels of mesothelin are correlated with a poor prognosis in cases with advanced malignancies, particularly KRAS-mutant lung adenocarcinoma [14]. Also, studies have shown that mesothelin may facilitate metastatic progression by enhancing peritoneal implantation and promoting cancer cell survival and proliferation [15]. These characteristics imply that mesothelin is a potential target for immunotherapy for cases with advanced malignancies [13].

Our previous preclinical studies using mouse models of gastric and ovarian tumors have demonstrated that MSLN-CAR-T cells can effectively inhibit the growth of tumors and prolong the survival of mice [16]. Previous clinical trials have shown that mesothelin-targeted CAR-T cell therapy is safe, but its clinical effects are very limited. For example, the clinical trial (NCT02159716) shows that the optimal clinical response to MSLN-CAR T cell therapy for advanced tumors is stable disease (11/15 patients), no partial or complete response. Therefore, improving CAR-T cell engineering is necessary in order to develop a better MSLN-CAR-T cell therapy for solid tumors [17,18].

The effects of CAR-T cell therapy were significantly restricted by the Immune Tumor Microenvironment (TME) [19]. Checkpoint ligands (including PD-L1) bind to checkpoint receptors (such as PD-1) on T cells to suppress the antitumor activity of T cells. Cancer cells suppress CAR-T cell immunity by upregulating the expression of checkpoint ligands (such as PD-L1), leading to tumor escape. It has been shown that CRISPR/Cas9-mediated disruption of the Programmed cell Death-1 (PD-1) gene locus in human CAR-T cells did not affect their proliferation but strongly augmented their cytokine production and cytotoxicity towards PD-L1-expressing cancer cells *in vitro* [20]. Tanoue et al. [21] demonstrated that oncolytic adenovirus expressing PD-L1 mini-body can enhance antitumor effects of CAR-T cells in solid tumors. This PD-L1 oncolytic strategy was shown to be more efficient in reinvigorating CAR-T cells compared to systemic injection of PD-L1 antibodies. John et al. [22] also showed that combining anti-Her-2 CAR-T cell therapy with anti-PD-1 antibody treatment significantly reduced the growth of breast carcinoma tumors in mice compared to anti-Her-2 CAR-T cell therapy alone or in combination with isotype control antibody. Fang et al. [23] reported a case of refractory epithelial ovarian cancer in a patient who had relapsed after multiline chemotherapy. This patient achieved partial response and survived more than 17 months after receiving αPD-1-MSLN-CAR-T cells therapy in addition to apatinib treatment.

Based on these observations, we hypothesized that the combination of anti-PD-1 antibody with mesothelin-targeted CAR-T cells could provide a potential therapeutic advantage [24]. In this study, we generated MSLN-CAR-T cells with the capacity to secret anti-PD-1 antibodies. An exploratory trial was performed to assess the safety of αPD-1-MSLN-CAR-T cells in advanced malignancies.

Materials and Methods

Patients

Adult patients (aged ≥ 18 years) with measurable lesions after the failure of two or more conventional treatments with chemotherapy or radiotherapy were enrolled in the trial. All patients met the inclusion standards with previous tumor specimens or new biopsies detected as MSLN and PD-L1 positive on pathological examination. Immunohistochemistry (IHC) was performed to evaluate the expression of MSLN and PD-L1 using Mesothelin (D9R5G) XP® Rabbit mAb (Abcam, Cambridge, England) at a 1:300 dilution and Anti-PD-L1 antibody (73-10) (Abcam, Cambridge, England) at a 1:600 dilution. The GTVision III Detection System/Mo&Rb Kit was used for color development. Images were obtained using an AXIOSTAR PLUS microscope (ZEISS, Jena, Germany). MSLN/PD-L1 expression was evaluated using a four-point scale: 0, no expression; 1+, weak expression; 2+, medium expression; 3+, strong expression. All patients had completed other cancer treatments at least 2 weeks before recruitment to our study. The ECOG scores of all patients were between 0 to 1.

Patients with active viral or bacterial infections, severe organ dysfunction medical histories including immunodeficient and systemic autoimmune diseases were excluded from the study. Patients that had been prescribed treatment with glucocorticoids or immunosuppressive agents one month before the investigation were also excluded. All cases signed the informed consent before treatment.

Research design

This study was an investigator-initiated trial that was conducted at the Suzhou BenQ Hospital (Suzhou, China) and the Fifth Ningbo People's Hospital (Ningbo, China). The protocol was approved by the Ethics Committee of the hospitals. Patients with MSLN and PD-L1 positive advanced malignancies were eligible to be enrolled on the study unless the patients met the exclusion criteria. Measurable tumor lesions were confirmed through radiologic examinations 7 days before cell infusion. Fludarabine (FLU) was prescribed at a dose of 25 mg/kg/day for lymphodepletion on 3 consecutive days. αPD-1-MSLN CAR-T cells were given to the patients through intravenous drip at a concentration of 2×10^6 cells/kg. After cell infusion, patients remained in hospital for 7 days of observations. Peripheral blood was collected according to the scheme of the infusion and for the assessment of cytokine levels, CAR-T copy numbers and anti-PD-1 antibody concentrations (Figure 2A).

αPD-1-MSLN-CAR-T design and cell manufacturing

The MSLN CAR gene encoding the mesothelin scFv with the CD28 co-stimulatory and CD3ζ endo-domains and the αPD-1 gene encoding the anti-PD-1 scFv with a Fc fragment of human Immunoglobulin G4 (IgG4) (Figure 1A) were cloned into the piggyBac transposon vectors pNB338B and pS338B, respectively. And these constructs were confirmed by DNA sequencing.

Peripheral Blood Mononuclear Cells (PBMCs) were separated from 120 ml of blood and purified utilizing the Ficoll density gradient. Cells were then cultured in CTS™ AIM-V™ Medium (Gibco, New York, USA) overnight in the absence of phenol red and antibiotics. PBMCs were collected and suspended in the electroporating buffer (Lonza, Basel, Switzerland) and mixed with the pNB338B-MSLN-CAR and pS338B-αPD-1 for electroporation using FI-115 program of Lonza 4D electroporator (Lonza, Basel, Switzerland) conforming to the instructions of the manufacturer. Following the electroporation, the cells were cultivated in the AIM-V medium including 5% CTS™ Immune Cell Serum Replacement (SR; Gibco, New York, USA) for four hours and then transferred into 6-well plates pre-coated with MSLN antigen (5 μg·mL^{-1})/anti-CD28 antibody (5 μg·mL-1). The cells were cultured for 4 to 5 days in a 5% SR-AIM-V medium containing 500 U/mL of recombinant human IL-2. Next, the cultivation of the activated cells was carried out in a 5% SR-AIM-V medium supplemented with 100 U/mL IL-2 for about 10 to 14 days

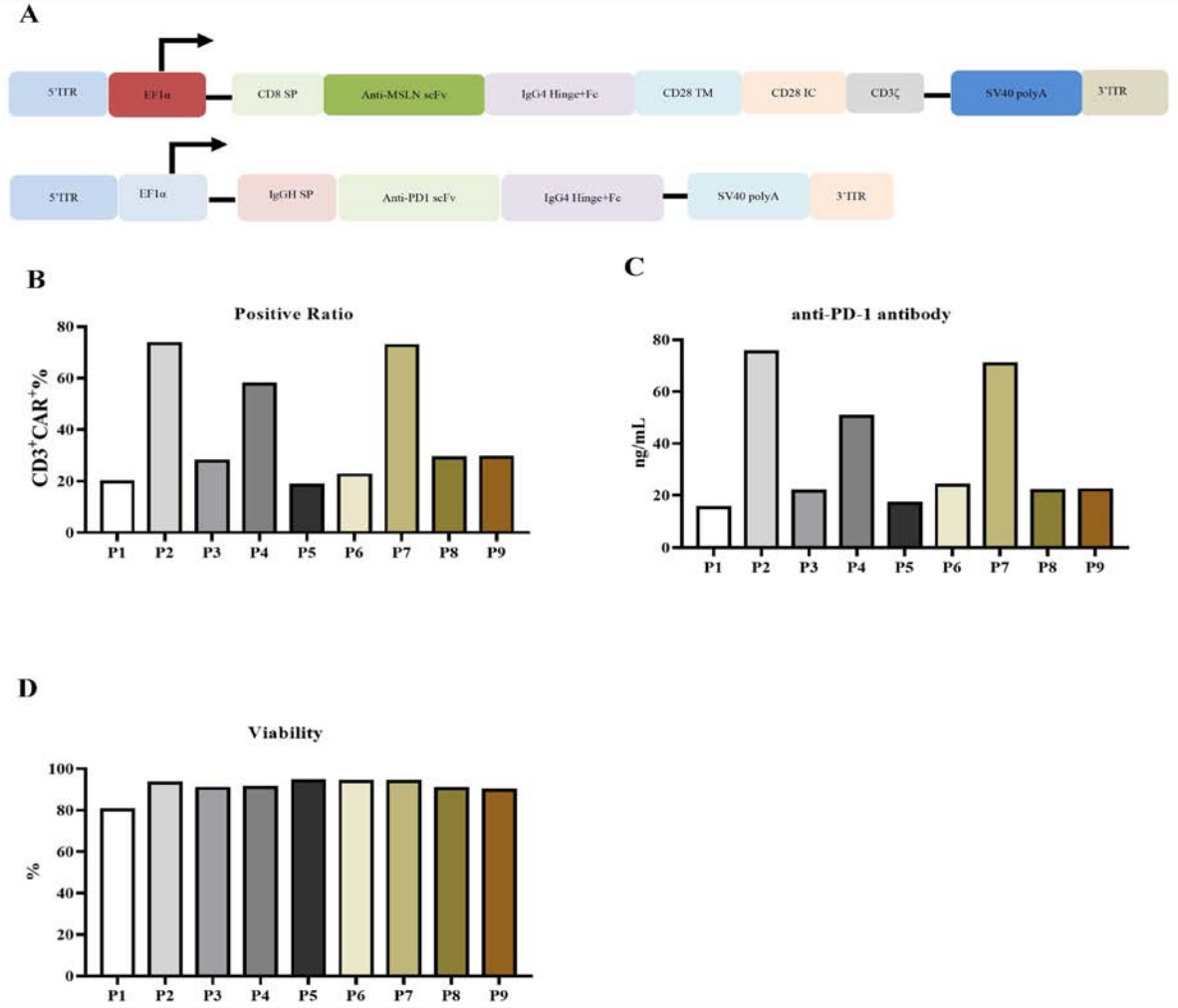


Figure 1: αPD-1-MSLN-CAR gene structure and the characteristics of αPD-1-MSLN-CAR-T cells. (A) The Schematic shows the MSLN-CAR gene encoding the anti-MSLN scFv with CD28 co-stimulatory and CD3ζ endo-domains. The schematic below shows the αPD-1 gene encoding the anti-PD-1 scFv with a Fc fragment of the human immunoglobulin G4 (IgG4). (B) Expression of MSLN-CAR in the T cells of the 9 patients in the study. (C) Anti-PD-1 antibody concentrations of T cells cultured *in vitro* were detected. (D) Cell viability analysis of the 9 patients in this study before infusion into patients.

Table 1: Characteristics of patients.

Patient No.	Sex	Age	Diagnosis/Stage	Metastasis	ECOG	Prior therapies	Latest Evaluation	IHC staining intensity score	
								MSLN	PD-L1
P1	M	78	NSCLC/IV	Lymph node,	0	Surgery, TP, Anlotinib, radiotherapy	PD	+++	++
				Bone					
P2	F	66	PDAC/IV	Liver	0	Paclitaxel + GE	PD	+++	+
P3	M	65	CRC/IV	Liver,	1	Surgery, FOLFOX,	PD	++	+
				Lymph node		FOLFIRI, bevacizumab			
P4	F	61	malignant pleural mesothelioma/IV	lung	1	pemetrexed + cis-platinum	PD	+++	+
P5	F	40		Lymph node	1	Paclitaxel+ cis-platinum, radiotherapy	PD	+++	++
			cervical cancer/III						
P6	F	80	NSCLC/IV	Cancerous hydrothorax, Lymph node	0	pemetrexed + cis-platinum, Anlotinib	PD	+++	+
P7	F	64	NSCLC/IV	Lung, Lymph node	1	Surgery, TP, radiotherapy	PD	+++	+
P8	M	46	gastric carcinoma/IV	enterocoelia, Lymph node	1	XELOX, Paclitaxel	PD	+++	+
P9	F	53	ovarian cancer/IV	enterocoelia, Lymph node	1	Surgery, Paclitaxel+ carboplatin, etoposide	PD	++	+

Abbreviations: NSCLC: Non-Small-Cell Lung Cancer; PDAC: Pancreatic Ductal Adenocarcinoma; CRC: Colorectal Carcinoma; PD: Progressive Disease; TP: Paclitaxel and Cis-Platinum; GE: Gemcitabine; FOLFOX: Oxaliplatin, Leucovorin and 5-FU; FOLFIRI: Irinotecan, Leucovorin and 5-FU; GP: Gemcitabine and Cis-Platinum; NX: Navelbine and Capecitabine; XELOX: Oxaliplatin and Capecitabine

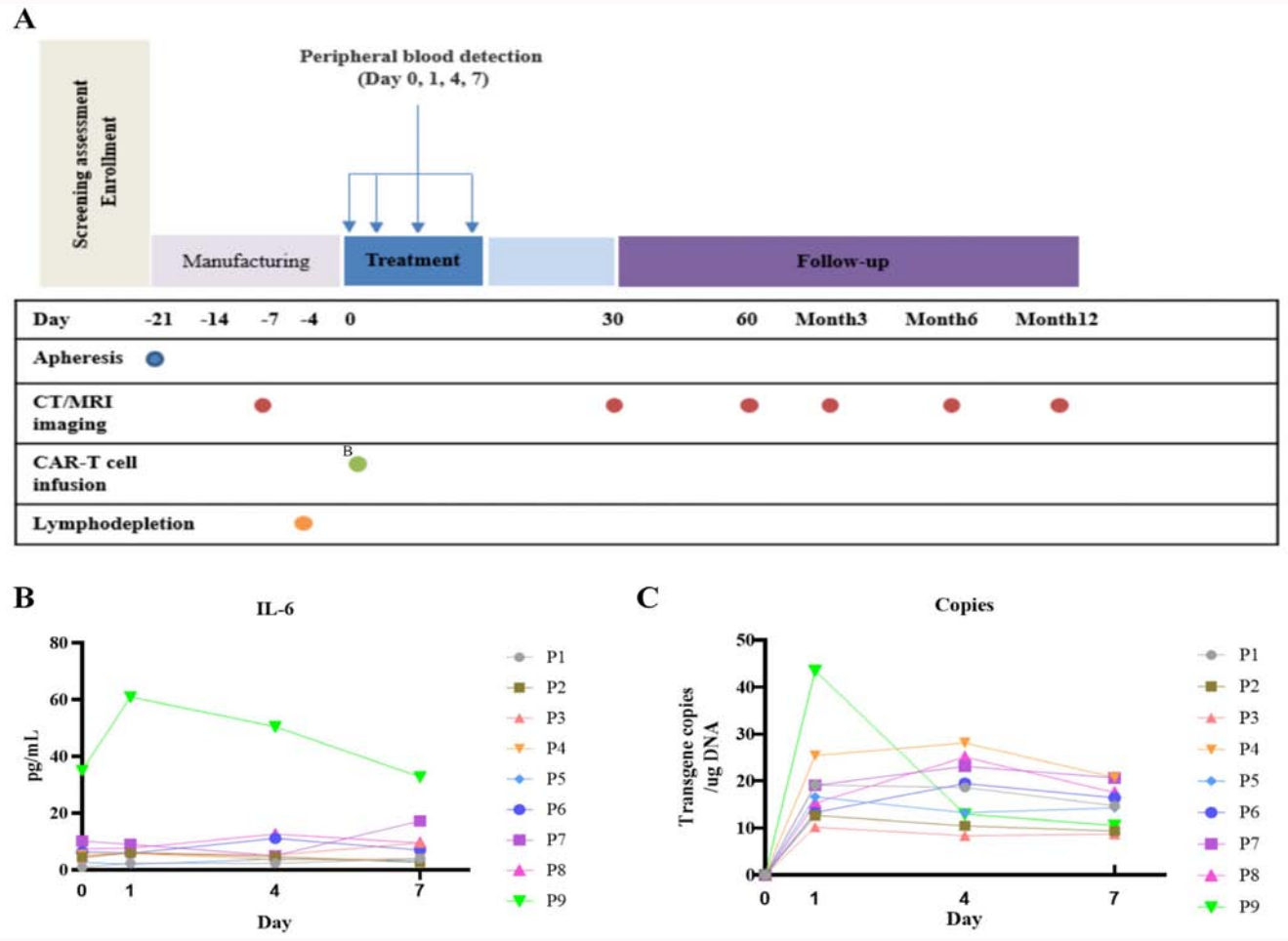


Figure 2: Study design and the persistence of αPD-1-MSLN-CAR T cells in peripheral blood. (A) The protocol for screening and the manufacturing of autologous αPD-1-MSLN-CAR-T cells, and lymphodepletion succeeded by infusion of αPD-1-MSLN-CAR-T cells and follow-up. (B) Detection of cytokines (IL-6) levels before treatment and at serial time points following the cell infusion. (C) Detection of CAR copy numbers prior to the treatment and at serial time points following the infusion of the cell.

to obtain sufficient quantities of cells. The quality of the CAR-T cells was determined by measuring their viability, the percentage of CAR-positive cells, and the expression of anti-PD-1 antibody using trypan blue dye exclusion method, flow cytometry, and ELISA.

Evaluation of T cell phenotype by flow cytometry

The CAR positive ratio of CAR-T cells was evaluated by flow cytometry using biotin-conjugated MSLN antigen plus PE-conjugated streptavidin (BD Biosciences, San Jose, CA, USA). The surface phenotype of αPD-1-MSLN-CAR-T cells was assayed with fluorescently-labeled antibodies specific for CD3, CD4, CD8, CD45RO, CD62L, and CCR7 (BD Biosciences). CD3+ central memory and effector memory cells were defined as CD45RO+CCR7+CD62L+ and CD45RO+CCR7-CD62L-, respectively. Flow cytometry detection was performed using a flow cytometer of Navios (Beckman Coulter, America) and the achieved outcomes were studied using the Kaluza computer program package.

Measurement of anti-PD-1 antibody by ELISA

The PD-1 antibody concentration of the CAR-T cell supernatant in vitro was determined by ELISA. CAR-T cells were seeded into 12-well plates at a density of 2×10^5 cells per well containing 1 ml medium and then collected after 24 h. Peripheral blood samples of all cases were collected and the anti-PD-1 antibody concentrations were measured. Measurements were performed using the corresponding anti-antibody ELISA kits according to the manufacturer's instructions. Briefly, a 96-well ELISA microplate coated with 0.5 μg/ml PD-1 protein (ACRO Biosystems, Beijing, China) was incubated at 4°C overnight. After the microplate was blocked with 1% BSA for 2 h at 37°C, the collected supernatant was added to the well and incubated for 1 h at 37°C. After washing the wells three times, mouse anti-human IgG4 conjugated HRP (Abcam, Cambridge, England) was added to the well at a dilution of 1:30,000 and incubated for 45 min at 37°C. After washing the wells five times, TMB reagents were added for color development. The color reaction was stopped with TMB solution, and the Optical Density (OD) at 450 nm measured using the Multiscan Spectrum (Perkin Elmer, Connecticut, USA).

T cell viability measured by trypan blue dye exclusion assay

10 ul of cell suspension was mixed with 0.4% trypan blue solution (Gibco, New York, USA). Cells were counted using Countess II FL Automated Cell Counter (Life Technologies, California, USA).

Assessment of serum cytokines through cytometric bead array

The levels of IL-2, IL-4, IL-6, IL-10, IFN-γ and TNF-α levels in serum were assessed using flow cytometry with a Cytometric Bead

Table 2: Subset composition of αPD-1MSLN-CAR-T cells the final products.

Patient No.	$CD3^+$ T cell (%)	$CD3^+$ CAR-T cell (%)	$CD3^+CD4^+$ CAR-T cell (%)	$CD3^+CD8^+$ CAR-T cell (%)	$CD45RO^+CCR7^+CD62L^+$ T cell (%)	$CD45RO^+CCR7^-CD62L^-$ T cell (%)
P1	77.42	19.71	2.69	20.41	6.88	31.61
P2	93.22	73.37	61.46	17.61	7.2	46.89
P3	96.09	27.73	11.57	17.98	58.97	5.53
P4	74.36	57.63	14.24	62.68	4.1	73.17
P5	72.51	18.41	4.06	19.12	7.67	55.31
P6	74.99	22.38	4.2	22.65	6.43	27.9
P7	84.81	72.72	16.57	62.68	10.87	31.29
P8	70.69	29.04	8.52	26.58	3.63	41.26
P9	91.97	29.04	11.43	19.53	38.37	17.05

Array (CBA) Human Th1/Th2 Cytokine Kit II (BD, America). Peripheral blood samples were collected from all the enrolled cases on days 0, 1, 4, 7 and all samples were analyzed.

Detection of MSLN-CAR transcripts *via* qPCR analysis

Genomic DNA was isolated from whole blood specimens and qPCR was used to measure MSLN-CAR-T levels. Transgene-specific primers and probes recognizing the CD28-CD3z junctional fragment in the intracellular signaling region of the CAR were synthesized or labeled by Shanghai Generay Biotech Co. Ltd (Shanghai, China). The sequences were as follows: Forward, 5'-CTCCTGCACAGTGACTACATG-3'; Reverse, 5'- GAACTTCACTCTGGAGCGATAG-3'; Probe, FAM-ccgCaaGcaTtaCcagcc-TAMRA.

Total DNA was extracted from samples using Genomic DNA Extraction Kit Ver.5.0 (TaKaRa, Japan). Real-time polymerase chain reaction was performed using the TaqMan™ Universal Master Mix II (Thermo Fish Scientific, Massachusetts, USA). The quantitative real-time PCR reaction was performed in two steps: (1) Pre-incubation: 95°C for 5 min; (2) Amplification: 40 cycles of 95°C for 20 sec followed by 60°C for 1 min. Each sample was analyzed in triplicate and the mean values were used for data analysis. The data were exported by LightCycler 480 II (Roche, Basel, Switzerland). MSLN-CAR-T levels were reported as the number of transgene copies per microgram of genomic DNA.

Safety assessments

All patients were evaluated for the efficacy administration of αPD-1-MSLN-CAR-T cells conforming to immune-related response criteria and Response Evaluation Criteria in RECIST 1.1. Further, the grade of the adverse events was accomplished conforming to the National Cancer Institute Common Terminology Criteria for Adverse Events (CTCAE) version 5.0. The incidence of treatment-related Adverse Events (AEs), clinical events and laboratory toxicities, were described as definitely, likely, or possible, corresponding to participation in the research.

In the study, AEs included toxicity of chemotherapy, infusion toxicity, and other types of systemic toxicities that could be related to αPD-1-MSLN-CAR-T cells. We defined Dose-Limiting Toxicities (DLT) events that were as a grade 3 or greater including non-hematologic or hematologic events that occurred up to a maximum of 28 days after the treatment and had not been recorded before αPD-1-MSLN-CAR-T cells infusion.

All DLTs were as grade 3 or higher events and defined as follows; non-hematological toxicities excluding asymptomatic electrolyte imbalance, fatigue, nausea, vomiting, and diarrhea, hematologic toxicities excluding asymptomatic lymphopenia or other preexisting blood counting events, and allergic reactions including hypersensitivities and autoimmune responses such as peritonitis, pleuritic and pericarditis.

Statistical analysis

Statistical analysis was carried out utilizing SPSS Statistical computer program 21. The results were presented as the means ± standard deviation, the medians with ranges and proportions. The survival and Progression-Free Survival (PFS) curves were analyzed using the Kaplan-Meier method. A P-value of <0.05 was considered statistically significant.

Results

Patient characteristics

From July 2018 to July 2019, 9 cases who met the inclusion benchmarks were recruited to the study. The clinical features of these cases are briefly described in Table 1. Six of the 9 cases were female, and the average age of the cases was 61.4 years (range 40 to 80 years). Of the 9 patients enrolled in the study, 3 had advanced lung adenocarcinoma, 1 had advanced colonic adenocarcinoma, 1 had advanced pancreatic cancer, 1 had Malignant Pleural Mesothelioma (MPM), 1 had advanced cervical squamous carcinoma, 1 had advanced ovarian papillary cystadenocarcinoma, and 1 had advanced gastric adenocarcinoma. Seven of 9 patients had a strong positive expression of mesothelin (+++), 2 of 9 patients had a medium positive expression of PD-L1 (++) (Supplemental Figure 1). All patients were diagnosed as stage III/IV and most of the patients had metastatic lesions in the lymph nodes, liver or lungs.

Characteristics of infused αPD-1-MSLN-CAR-T cells

Autologous PBMCs were transduced with CAR encoding scFv for mesothelin and anti-PD-1 antibody. Before infusing the T cells into patients, we measured the CAR positive ratio, the production capacity of PD-1 antibody, and the activity of T cells. The average CAR positive ratio was 38.91% and ranged from 18.41% to 73.37% (Figure 1B); the average proportion of CD3+/CD4+ T cells was 14.97% (2.69-61.46%) and the average proportion of CD3+/CD8+ T cells 29.92% (17.61-62.68%). The average proportions of central memory and effector memory T cells was 16.02% (3.63-58.97%) and 36.67% (5.53-73.17%), respectively (Table 2). Anti-PD-1 antibody concentrations were measured by ELISA. The average concentration of anti-PD-1 antibody in CAR-T products was 35.32 ng/ml and range from 15.31 ng/ml to 70.60 ng/ml (Figure 1C). All of the infused T cells from different patients showed high viability. The average viability of T cells was 90.70% (80.11% to 94.1%) (Figure 1D).

Table 3: Clinical effect of αPD-1 MSLN-CAR-T cells treatment.

NO. of patients	Objective Response					NO. (%) of patients		Survival, months (95% CI)	
	CR	PR	SD	PD	NE	ORR	DCR	PFS	OS
14	0	2	4	3	0	2/9(22.2%)	6/9(66.7%)	4.2 (2.7–5.7)	6.5 (4.7–8.3)

Abbreviations: CR: Complete Response; PR: Partial Response; SD: Stable Disease; PD: Progressive Disease; NE: Not Evaluated; ORR: Objective Response Rate (CR And PR); DCR: Disease Control Rate (CR, PR, and SD); PFS: Progression-Free Survival; OS: Overall Survival

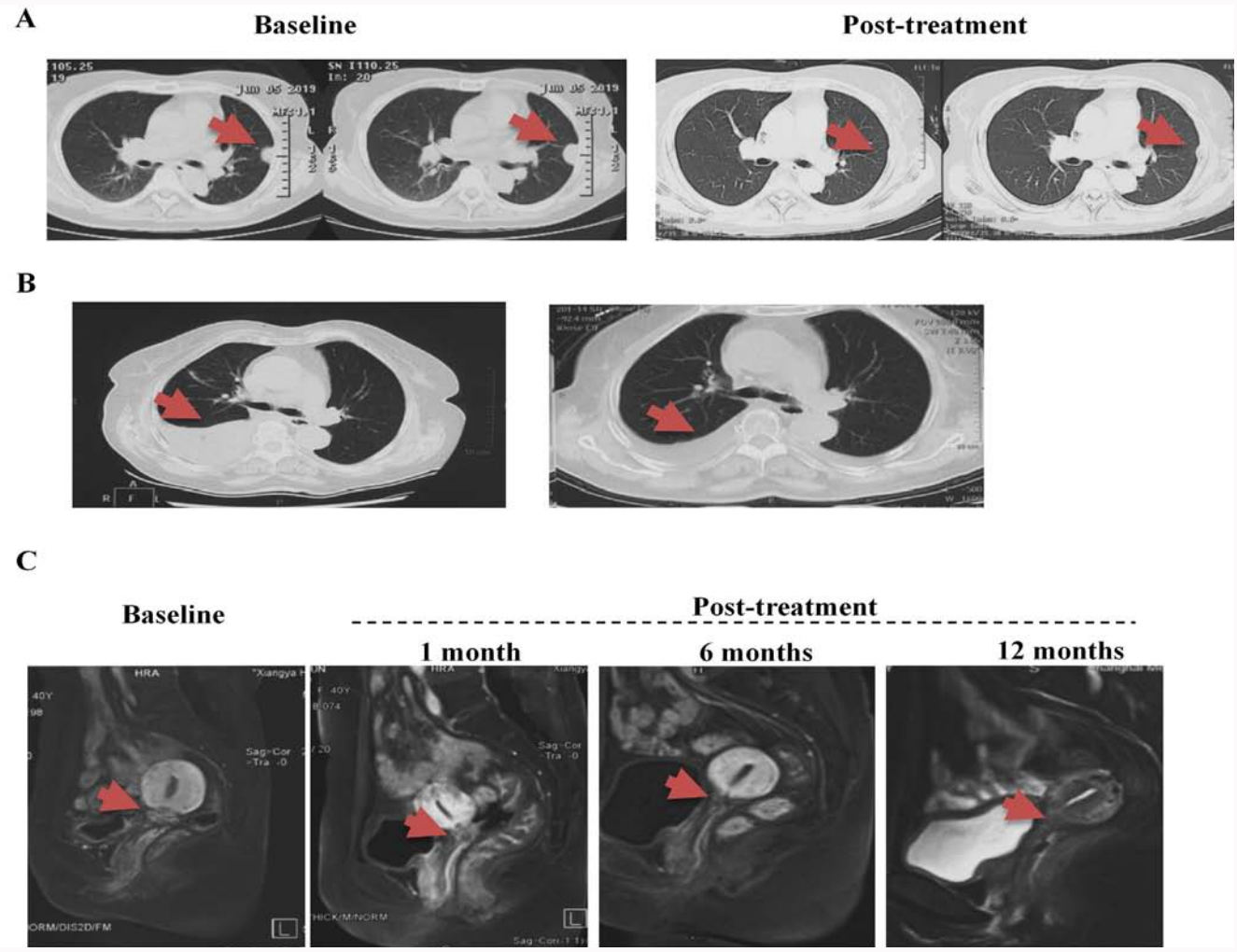


Figure 3: The clinical responses of αPD-1-MSLN-CAR-T Cells Therapy. (A) Chest Computed Tomography (CT) assessment of P4 before and 1 month after αPD-1-MSLN-CAR-T cells infusion. (B) Chest Computed Tomography (CT) assessment of P6 before and 1 month after αPD-1-MSLN-CAR-T cells infusion. (C) Pelvic MRI assessment of P5 before and 1 month, 6 months, 12 months following the infusion of αPD-1-MSLN-CAR-T cells.

Bioactivity of αPD-1-MSLN-CAR T-cells

Plasma cytokines levels were measured before and after cell infusion as shown in Figure 2A. Six cytokines (IL2, IL4, IL6, IL10, TNF-α, IFN-γ) were detected on a Chip array. No significant changes in the levels of IL2, IL4, IL10, TNF-α, IFN-γ were observed during αPD-1-MSLN-CAR-T cell treatment (data not shown). The levels of IL6 changed dramatically in 1 patient (P9). The highest level of IL6 was 60.99 pg/ml in P9 (Figure 2B). The production of anti-PD-1 antibodies was less than 1 ng/mL in 9 patients.

Expansion and persistence of αPD-1-MSLN-CAR-T cells *in vivo*

To evaluate the expansion and persistence of αPD-1-MSLN-CAR T cells, we analyzed CAR copy numbers by quantitative Polymerase Chain Reaction (qPCR) within 7 days after infusion (Figure 2C). We detected αPD-1-MSLN-CAR T cells in the peripheral blood of 9 cases and observed that most patients had a peak level of DNA copies after 1 day following each infusion.

Clinical activity of αPD-1-MSLN-CAR-T cells

The clinical response to αPD-1-MSLN-CAR T-cell therapy was determined by CT/MRI four weeks after the cell infusion. Among the 9 patients, 2 (P4, P5) achieved Partial Response (PR), 4 (P2, P6, P7 and P9) achieved Stable Disease (SD) from 3.7 months to 10.6 months, and 3 had Progressive Disease (PD). A chest CT of Patient 4 with malignant mesothelioma and multiple pulmonary metastases showed that the metastatic lesion in the lung regressed as a PR (Figure 3A). Chest CT of Patient 6 who had a right lung adenocarcinoma with cancerous hydrothorax is shown in Figure 3B. This patient showed reduced pleural effusion in the right lung after treatment. Pelvic MRI

Table 4: Patients' toxicity and patients' response.

Patient No.	Cytopenia (AE grade)	CRS-related events (AE grade)	CRS grade	Allergic reaction (AE grade)	Use of glucocorticoid	Response	PFS (months)	OS (months)
P1	lymphocyte count decreased (4)	No	0	No	No	PD	1	5.9
P2	lymphocyte count decreased (4)	No	0	No	No	SD	4.5	5.8
P3	lymphocyte count decreased (3)	No	0	No	No	PD	1	4.5
P4	lymphocyte count decreased (4), WBC decreased (3)	No	0	No	No	PR	12.5	16.1+
P5	lymphocyte count decreased (4), WBC decreased (3)	No	0	No	No	PR	19.5+	19.5+
P6	lymphocyte count decreased (3), WBC decreased (2)	No	0	No	No	SD	10.6	15.3
P7	lymphocyte count decreased (4)	No	0	No	No	SD	4.2	4.2
P8	lymphocyte count decreased (4)	No	0	No	No	PD	1	6.5
P9	lymphocyte count decreased (3), WBC decreased (1)	Fever (1)	1	No	No	SD	3.7	8.8

Abbreviations: WBC: White Blood Cell Count; CRS: Cytokine Release Syndrome; PFS: Progression-Free Survival; OS: Overall Survival; PR: Partial Response; SD: Stable Disease; PD: Progressive Disease

NOTE: AE grading and CRS grade were assessed using the National Cancer Institute Common Terminology Criteria for Adverse Events (CTCAE) version 5.0.

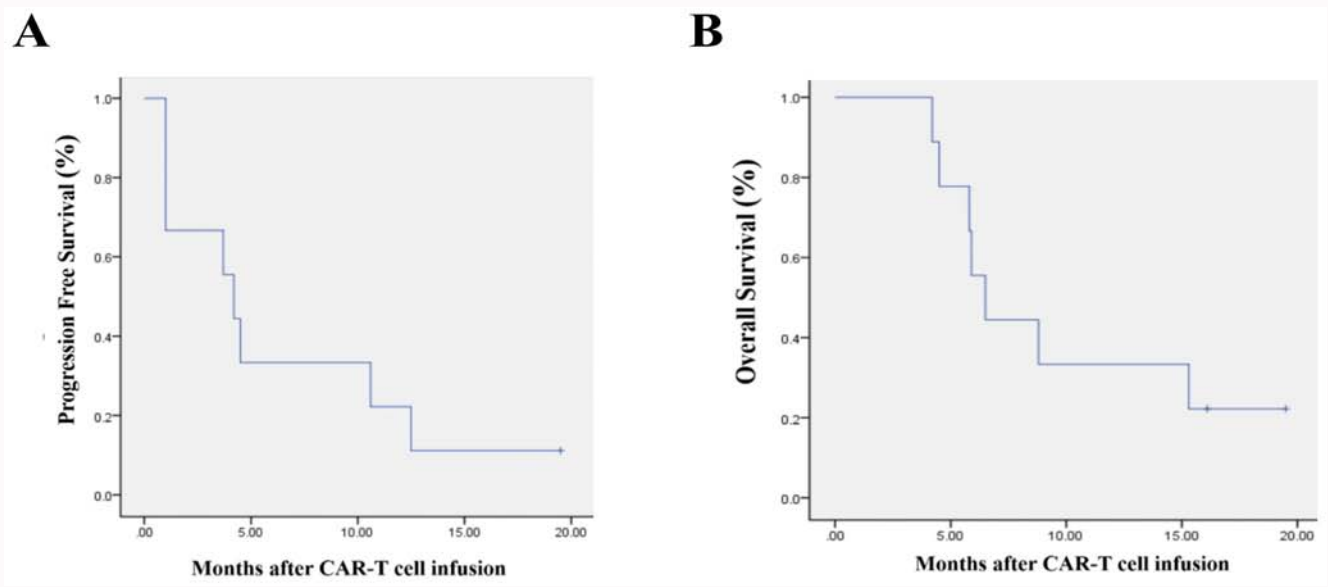


Figure 4: PFS and OS of the cases with αPD-1-MSLN-CAR-T therapy. (A) Progression-free survival (B) Overall survival *via* the Kaplan-Meier approach.

of Patient 5 who had cervical squamous carcinoma with lymphatic metastasis showed a reduction of the cervical uteri cancer at one month. Six months later, the tumor had dramatically regressed and had almost disappeared at 12 months (Figure 3C) and was assessed as a PR.

The Objective Response Rate (ORR) in all 9 patients was 22.2%; the Disease Control Rate (DCR) in all 9 patients was 66.6% (Table 3). The median Progression-Free Survival Time (PFS) was 4.2 months (95% CI, 2.7–5.7 months) (Figure 4A), and the median Overall Survival (OS) was 6.5 months (95% CI, 4.7–8.3 months) (Figure 4B).

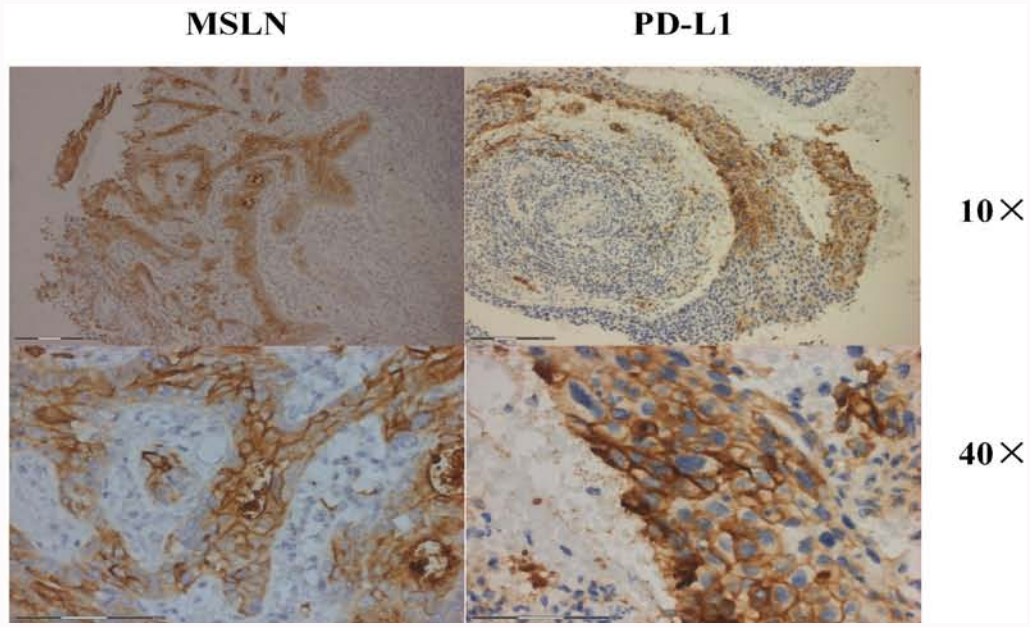
Safety of piggyBac-transduced αPD-1-MSLN-CAR-T cell therapy

In the current investigation of αPD-1-MSLN-CAR-T infusion in 9 patients. Safety evaluation was performed on day 28 after infusion. The adverse events relevant to the treatment are briefly described in Table 4. No neurotoxic adverse events were observed. Most patients experienced a grade 3/4 lymphodepletion. 1 patient (P9) had grade 1 fever (38.0 to 39.0°C) within 12 h after each cell infusion. Non-steroidal Anti-Inflammatory Drug (NSAID) like ibuprofen and physical cooling were used to relieve the fever in the patient.

Discussion

Current conventional treatments for most solid tumors (chemotherapy, radiotherapy, targeted therapy) have major side effects and are prone to the development of drug resistance. In the past 10 years, immunotherapy using CAR-T cells and immune checkpoint inhibitors have shown major efficacy in the anticancer treatments [25-27]. In the present clinical trial, we obtained data demonstrating the safety, feasibility, and effect of αPD-1-MSLN-CAR-T cells in cases with advanced relapsed and metastatic tumors. Previous studies have shown that MPM patients have a poor prognosis with a median survival of 8 to 14 months and a 5-year survival rate of less than 5% [28, 29]. In this study, it was particularly encouraging that 1 MPM patient receiving αPD-1-MSLN-CAR-T cells therapy had a sustained remission for more than 12 months. These observations indicate that αPD-1-MSLN-CAR-T cells therapy could be an effective treatment for MPM patients who have failed conventional therapies.

Current methods used to introduce CAR gene into primary T cells fall into two broad categories, viral gene transfection and non-



Supplemental Figure 1: Immunohistochemical analysis of P5 case cancer tissues for MSLN and PD-L1 expression.

viral-based DNA transfection [30,31]. The vast majority of clinical-grade CAR-T products are transfected with virus vectors because of their ability to effectively traverse cellular membranes during infection. However, viral vector systems also have some limitations including the potential for insertional mutagenesis and genotoxicity of viral vectors and high costs correlated with constructing clinical-grade viral vectors [32]. In comparison to the viral vector systems, the non-viral piggyBac transposon system has several advantages in the engineering of CAR-T cells including low-immunogenicity and low levels of genotoxicity, highly stable levels of gene transfection and uncomplicated, cost-effective production methods [31,33-35]. These advantages further ensure the safety of CAR-T cell products generated utilizing the system of piggyBac transposon for clinical applications.

Mesothelin is an interesting target for CAR-T cell therapy as it is expressed at low levels in normal mesothelial cells but highly expressed in a wide spectrum of solid tumors, including mesothelioma, pancreatic cancers, ovarian cancers, and lung cancer [11,12]. Our previous preclinical studies have shown that piggyBac-generated MSLN-CAR-T cell products have potential efficacy in the treatment of MSLN-positive pancreatic cancer and bile duct carcinoma *in vitro* and mouse xenografts [31,36]. Based on these data, we chose mesothelin as a target in this exploratory clinical trial.

Several clinical investigations have shown that anti-PD-1 antibody therapy can benefit cases with solid tumors. However, the target response rates to anti-PD-1 antibody therapy are not particularly high in patients with solid tumors [37]. Moreover, anti-PD-1 antibody treatment requires repeated intravenous administration to maintain the durable responses, increasing the incidence of systemic off-target immune-related toxicities [24,38]. The researchers demonstrated that anti-PD-1 antibodies can rescue the effector function of exhausted CAR-T cells, further enhancing the efficacy of CAR-T Cells therapy [39]. Considering the above limitations associated with anti-PD-1 antibody therapy, we designed MSLN CAR-T cells secreting PD-1 antibodies to promote accumulation of anti-PD-1 antibodies in the tumor microenvironment.

In the present study, we noticed that most patients developed hematologic toxicities, specifically grade 4 lymphocytopenia due to treatment with fludarabine that is utilized prior to CAR-T cell processing. However, these toxic events were acute and patients recovered in the short term. The toxic impacts potentially correlated with the CAR-T cell therapy include fever, off-target toxicity of tumor and anaphylaxis. No neurotoxicity or grade 3/4 cytokine release syndrome adverse events were observed.

In this study, 6 patients achieved disease control, of which 4 cases attained stable disease stability and 2 patients showed a partial reaction. The patients with stable disease tended to develop disease progression within 4 months. These findings suggest that further improvements are needed to optimize the antitumor impacts of αPD-1-MSLN-CAR-T cells in the treatment of advanced cancers. The limited levels of anticancer activity observed in this study may be due to several main reasons [40-43]. These include the high tumor burden in cases with advanced tumors, the restricted persistence and proliferation of CAR T cells, inappropriate trafficking of CAR T cells to the site of tumors, difficulty of T cell infiltration in tumor sites, and tumor antigen heterogeneity.

Previous clinical researches have shown that CAR-T cells are more effective in patients with low tumor burden compared to cases with a high tumor burden. Patients receiving CAR-T cell therapy in the early stage of the disease also show better anti-tumor effects [40,44,45]. In cases with a high tumor burden, chemotherapy drugs were selected according to the specific cancer indications and combined with fludarabine before treatment to reduce tumor burden and prevent lymphodepletion. The optimum dosing of chemotherapy also requires further exploration.

In this study, anti-PD-1 antibodies were not detected in the peripheral blood samples of most patients. Consistently, previously study showed that the anti-PD-1 antibody secreted by CAR-T cells hardly could be detected in the sera of mice [19]. We speculate that the

infused CAR-T cells were prone to homing to tumor tissues because of the attraction of tumor target antigen, so that the lower level of CAR-T cells and anti-PD-1 could be detected in the peripheral blood. αPD-1-MSLN-CAR-T cells were designed to produce anti-PD-1 antibodies in the tumor microenvironment to avoid immune-related toxic reactions caused by excessive systemic anti-PD-1 antibody concentrations. However, due to the lack of biopsy tissue after cell infusion, this hypothesis needs validation in further clinical trials.

For the better efficacy, our recent study indicated that a T cell-specific promoter could be used to improve the secretion of anti-PD-1 antibodies at the tumor sites [46].

In addition, the CAR-T cells secreting camelid nanobody (single variable domain of heavy chain (VHH) antibody fragments) had been shown to have better anticancer activities and persistence. VHHs have a low molecular weight and low immunogenicity and so genetically modified T cells can simultaneously secrete multiple nanobodies. VHH can replace murine-derived single-chain variable Fragments (scFv) to avoid immunological response and the disappearance of CAR-T cells [47,48]. In future studies, we will conduct exploratory clinical trials of MSLN-CAR-T cells secreting camelid nanobody for the cancer treatment. Patients will be eligible to receive multiple CAR-T cell infusions to maintain CAR-T cell levels if the processing is well tolerated and shows no adverse toxicities.

In summary, adoptive immunotherapy with αPD-1-MSLN-CAR-T cells generated using the piggyBac transposon system is a feasible and potential effective treatment for solid tumors. Our data suggest that αPD-1-MSLN-CAR T cell therapy has reduced side effects compared to chemotherapy and can provide an alternative treatment option for patients with advanced tumors. The current clinical trial data are useful towards informing the design of subsequent clinical trials aiming to assess the anticancer activity of αPD-1-MSLN-CAR T cells.

Funding

This study was supported by Shanghai Cell Therapy Group CO., LTD.

References

1. Ramos CA, Gianpietro D. Chimeric Antigen Receptor (CAR)- engineered lymphocytes for cancer therapy. Expert Opin Biol Ther. 2011;11(7):855-73.
2. Li S, Zhang J, Wang M, Fu G, Li Y, Pei L, et al. Treatment of acute lymphoblastic leukaemia with the second generation of CD19 CAR-T containing either CD28 or 4-1BB. Br J Haematol. 2018;181(3):360-71.
3. Pehlivan KC, Duncan BB, Lee DW. CAR-T cell therapy for acute lymphoblastic leukemia: Transforming the treatment of relapsed and refractory disease. Curr Hematol Malig Rep. 2018;13(5):396-406.
4. Abramson JS. Anti-CD19 CAR T-cell therapy for B-cell non-Hodgkin lymphoma. Transfus Med Rev. 2020;34(1):29-33.
5. Yan Z, Cao J, Cheng H, Qiao J, Zhang H, Wang Y, et al. A combination of humanised anti-CD19 and anti-BCMA CAR T cells in patients with relapsed or refractory multiple myeloma: A single-arm, phase 2 trial. Lancet Haematol. 2019;6(10):e521-9.
6. Beatty GL, O'Hara MH, Lacey SF, Torigian DA, Nazimuddin F, Chen F, et al. Activity of Mesothelin-specific chimeric antigen receptor T cells against pancreatic carcinoma metastases in a phase 1 trial. Gastroenterology. 2018;155(1):29-32.
7. Shi D, Shi Y, Kaseb AO, Qi X, Zhang Y, Chi J, et al. Chimeric antigen receptor-glypican-3 T-cell therapy for advanced hepatocellular carcinoma: Results of phase I trials. Clin Cancer Res. 2020;26(15):3979-89.
8. Junghans RP, Ma Q, Rathore R, Gomes EM, Bais AJ, Lo ASY, et al. Phase I trial of anti-PSMA designer CAR-T cells in prostate cancer: Possible role for interacting interleukin 2-T cell pharmacodynamics as a determinant of clinical response. Prostate. 2016;76(14):1257-70.
9. Brown CE, Alizadeh D, Starr R, Weng L, Wagner JR, Naranjo A, et al. Regression of glioblastoma after chimeric antigen receptor T-cell therapy. N Engl J Med. 2016;375(26):2561-9.
10. Ye L, Lou Y, Lu L, Fan X. Mesothelin-targeted second generation CAR-T cells inhibit growth of mesothelin-expressing tumors *in vivo*. Exp Ther Med. 2019;17(1):739-47.
11. Hara MO, Stashwick C, Haas AR, Tanyi JL. Mesothelin as a target for chimeric antigen receptor-modified T cells as anticancer therapy. Immunotherapy. 2016;8(4):449-60.
12. Morello A, Sadelain M, Adusumilli PS. Mesothelin-targeted CARs: Driving T cells to solid tumors. Cancer Discov. 2016;6(2):133-46.
13. Lv J, Li P. Mesothelin as a biomarker for targeted therapy. Biomark Res. 2019;7:18.
14. Thomas A, Chen Y, Steinberg SM, Luo J, Pack S, Raffeld M, et al. High mesothelin expression in advanced lung adenocarcinoma is associated with KRAS mutations and a poor prognosis. Oncotarget. 2015;6(13):11694-703.
15. Tang Z, Qian M, Ho M. The role of mesothelin in tumor progression and targeted therapy. Anti-cancer Agents Med Chem. 2013;13(2):276-80.
16. Zhang Z, Jiang D, Yang H, He Z, Liu X, Qin W, et al. Modified CAR T cells targeting membrane-proximal epitope of mesothelin enhances the antitumor function against large solid tumor. Cell Death Dis. 2019;10(7):476.
17. Klampatsa A, Dimou V, Albelda SM. Mesothelin-targeted CAR-T cell therapy for solid tumors. Expert Opin Biol Ther. 2021;21(4):473-86.
18. Haas AR, Tanyi JL, O'Hara MH, Gladney WL, Lacey SF, Torigian DA, et al. Phase I study of lentiviral-transduced chimeric antigen receptor-modified T cells recognizing mesothelin in advanced solid cancers. Mol Ther. 2019;27(11):1919-29.
19. Li Si, Siriwon N, Zhang X, Yang S, Jin T, He F, et al. Enhanced cancer immunotherapy by chimeric antigen receptor-modified T cells engineered to secrete checkpoint inhibitors. Clin Cancer Res. 2017;23(22):6982-92.
20. Hu W, Zi Z, Jin Y, Li G, Shao K, Cai Q, et al. CRISPR/Cas9-mediated PD-1 disruption enhances human mesothelin-targeted CAR T cell effector functions. Cancer Immunol Immunother. 2019;68(3):365-77.
21. Tanoue K, Rosewell SA, Watanabe N, Porter C, Rana B, Gottschalk S, et al. Armed oncolytic adenovirus expressing PD-L1 mini-body enhances antitumor effects of chimeric antigen receptor T-cells in solid tumors. Cancer Res. 2017;77(8):2040-51.
22. John LB, Devaud C, Duong CP, Yong CS, Beavis PA, Haynes NM, et al. Anti-PD-1 antibody therapy potently enhances the eradication of established tumors by gene-modified T cells. Clin Cancer Res. 2013;19(20):5636-46.
23. Fang J, Ding N, Guo X, Sun Y, Zhang Z, Xie B, et al. αPD-1-mesoCAR-T cells partially inhibit the growth of advanced/refractory ovarian cancer in a patient along with daily apatinib. J Immunother Cancer. 2021;9(2):e001162.
24. Grosser R, Cherkassky L, Chintala N, Adusumilli PS. Combination immunotherapy with CAR T cells and checkpoint blockade for the treatment of solid tumors. Cancer Cell. 2019;36(5):471-82.
25. Pardoll DM. The blockade of immune checkpoints in cancer immunotherapy. Nat Rev Cancer. 2012;12(4):252-64.
26. Buchbinder EI, Desai A. CTLA-4 and PD-1 pathways: Similarities, differences, and implications of their inhibition. Am J Clin Oncol. 2016;39(1):98-106.

27. Chow VA, Shadman M, AK G. Translating anti-CD19 CAR T-cell therapy into clinical practice for relapsed refractory diffuse large B-cell lymphoma. Blood. 2018;132(8):777-81.

28. Bibby AC, Tsim S, Kanellakis N, Ball H, Talbot DC, Blyth KG, et al. Malignant pleural mesothelioma: An update on investigation, diagnosis and treatment. Eur Respir Rev. 2016;25(142):472-86.

29. Bakker E, Guazzelli A, Ashtiani F, Demonacos C, Krstic-Demonacos M, Mutti L, et al. Immunotherapy advances for mesothelioma treatment. Expert Rev Anticancer Ther. 2017;17(9):799-814.

30. Dai H, Wang Y, Lu X, Han W. Chimeric antigen receptors modified T-cells for cancer therapy. J Natl Cancer Inst. 2016;108(7):djv439.

31. He J, Zhang Z, Lv S, Liu X, Cui L, Jiang D. Engineered CAR T cells targeting mesothelin by piggyBac transposon system for the treatment of pancreatic cancer. Cell Immunol. 2018;329:31-40.

32. Hudecek M, Izsvák Z, Johnen S. Going non-viral: The sleeping beauty transposon system breaks on through to the clinical side. Crit Rev Biochem Mol Biol. 2017;52(4):355-80.

33. Li X, Burnight ER, Cooney AL, Malani N, Brady T, Sander JD, et al. piggyBac transposase tools for genome engineering. Proc Natl Acad Sci USA. 2013;110(25):E2279-87.

34. Ramanayake S, Bilmon I, Bishop D, Dubosq M-C, Blyth E, Clancy L, et al. Low-cost generation of good manufacturing practice-grade CD19-specific chimeric antigen receptor-expressing T cells using piggyBac gene transfer and patient-derived materials. Cytotherapy. 2015;17(9):1251-67.

35. Woodard LE, Wilson MH. piggyBac-ing models and new therapeutic strategies. Trends Biotechnol. 2015;33(9):525-33.

36. Xu JY, Ye ZL, Jiang DQ, He J-C, Ding Y-M, Li L-F, et al. Mesothelin-targeting chimeric antigen receptor-modified T cells by piggyBac transposon system suppress the growth of bile duct carcinoma. Tumour Biol. 2017;39(4):1010428317695949.

37. Topalian SL, Hodi FS, Brahmer JR, Gettinger SN, Smith DC, McDermott DF, et al. Safety, activity, and immune correlates of anti–PD-1 antibody in cancer. N Engl J Med. 2012;366(26):2443-54.

38. Hamanishi J, Mandai M, Matsumura N, Abiko K, Baba T, Konishi I, et al. PD-1/PD-L1 blockade in cancer treatment: Perspectives and issues. Int J Clin Oncol. 2016;21(3):462-73.

39. Cherkassky L, Morello A, Villena-Vargas J, Feng Y, Dimitrov DS, Jones DR, et al. Human CAR T cells with cell-intrinsic PD-1 checkpoint blockade resist tumor-mediated inhibition. J Clin Invest. 2016;126(8):3130-44.

40. Wang Y, Chen M, Wu Z, Tong C, Dai H, Guo Y, et al. CD133-directed CAR T cells for advanced metastasis malignancies: A phase I trial. OncoImmunology. 2018;7(7):e1440169.

41. Iwai Y, Hamanishi J, Chamoto K, Honjo T. Cancer immunotherapies targeting the PD-1 signaling pathway. J Biomed Sci. 2017;24(1):26.

42. Knochelmann HM, Smith AS, Dwyer CJ. CAR T cells in solid tumors: Blueprints for building effective therapies. Front Immunol. 2018;9:1740.

43. Ma S, Li X, Wang X, Cheng L, Li Z, Zhang C, et al. Current progress in CAR-T cell therapy for solid tumors. Int J Biol Sci. 2019;15(12):2548-60.

44. Dai H, Zhang W, Li X, Han Q, Guo Y, Zhang Y, et al. Tolerance and efficacy of autologous or donor-derived T cells expressing CD19 chimeric antigen receptors in adult B-ALL with extramedullary leukemia. Oncoimmunology. 2015;4(11):e1027469.

45. Wang Y, Zhang WY, Han QW, Liu Y, Dai H-R, Guo Y-l, et al. Effective response and delayed toxicities of refractory advanced diffuse large B-cell lymphoma treated by CD20-directed chimeric antigen receptor-modified T cells. Clin Immunol. 2014;155(2):160-75.

46. Fang Y, Zhang Y, Guo C, Zhou X, Liu T, Qian Q, et al. Safety and efficacy of an immune cell-specific chimeric promoter in regulating anti-PD-1 antibody expression in CAR T cells. Mol Ther Methods Clin Dev. 2020;19:14-23.

47. Xie YJ, Dougan M, Ingram JR, Pishesha N, Fang T, Momin N, et al. Improved antitumor efficacy of chimeric antigen receptor T cells that secrete single-domain antibody fragments. Cancer Immunol Res. 2020;8(4):518-29.

48. Rahbarizadeh F, Ahmadvand D, Moghimi SM. CAR T-cell bioengineering: Single variable domain of heavy chain antibody targeted CARs. Adv Drug Deliv Rev. 2019;141:41-6.