Clinical Cancer Research

Dendritic Cell/Cytokine-Induced Killer Cell Immunotherapy Combined with S-1 in Patients with Advanced Pancreatic Cancer: A Prospective Study

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Abstract

Purpose: Advanced pancreatic cancer has remained challenging to treat effectively. This study aimed to investigate the clinical effects and safety of immunotherapy with dendritic cells and cytokine-induced killer cells (DC-CIK) administered with the chemotherapy (CT) S-1 in this malignancy.

Experimental Design: Consecutive patients ($n = 47$) with advanced pancreatic cancer were treated with either DC-CIK + S-1, DC-CIK alone, S-1 alone, or best supportive care.

Results: DC-CIK plus S-1 produced significantly longer median OS and PFS (212 and 136 days) compared with DC-CIK (128 and 85 days), CT (141 and 92 days), or supportive care only (52 and 43 days; $P < 0.001$). After adjusting for competing risk factors, DC-CIK combined with S-1 and receipt of 2 or more cycles of DC-CIK treatment remained independent predictors of disease-free and overall survival ($P < 0.05$). Phenotypic analysis of PBMCs demonstrated that the $CD3^+$, $CD3^+/CD4^+$, and $CD8^+/CD28^+$ T-cell subsets were elevated ($P < 0.05$), while the $CD3^+/CD8^+$, $CD3^+/CD16^+/CD56^+$ and $CD4^+/CD25^+$ cell subsets were significantly decreased after DC-CIK cell therapy ($P < 0.05$). There were no grade 3 or 4 toxicities. In addition, the mutational frequency in cell-free tumor DNA (cfDNA) declined in 4 of 14 patients who received DC-CIK, and was associated with a more favorable survival.

Conclusions: Treatment of advanced pancreatic cancer with combined DC-CIK infusions and S-1 was safe, resulted in favorable PFS and OS, and modulated the peripheral blood immune repertoire. *Clin Cancer Res; 23(17); 5066–73. ©2017 AACR.*

Introduction

Pancreatic cancer has remained challenging to treat with few patients eligible for resection and median survivals of 6–12 months for those with metastatic diseases, despite use of multiagent chemotherapy (1, 2). New therapeutic modalities that can synergize with existing chemotherapies without increased toxicity are critically needed. One such strategy is immunotherapy combined with well-tolerated chemotherapies.

S-1 (Taiho Pharmaceutical Company), an oral combination of the fluoropyrimidine tegafur, the DPD inhibitor gimeracil, and oteracil potassium, intended to reduce gastrointestinal toxicity of fluorouracil, has shown efficacy in various gastrointestinal malignancies (3–5). Phase II studies of S-1 as first-line therapy for metastatic pancreatic cancer resulted in response rates of 21.1% to 37.5% (6). Monotherapy with S-1 was noninferior to gemcitabine in overall survival with good tolerability (7). Moreover, in gemcitabine-refractory patients, a phase II study of S-1 reported a response rate (RR) of 15%, a median PFS of 2.0 months, and a median overall survival (OS) of 4.5 months (8). S-1 thus presents a convenient oral alternative for locally advanced and metastatic pancreatic cancer.

Recently, adoptive cell immunotherapy, delivery of *ex vivo* activated cellular products such as dendritic, natural killer (NK), or T cells, has garnered more attention as a treatment option for many kinds of malignancies, including advanced pancreatic cancer (9, 10). Dendritic cells (DC), are potent stimulators of tumor-specific T-cell responses. Cytokine-induced killer (CIK) cells are *ex vivo*–expanded lymphocytes with a NK/T-cell phenotype (expressing both CD56 and CD3),

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Translational Relevance

Advanced pancreatic cancer (APC) has remained challenging to treat effectively. New therapeutic modalities that can synergize with existing chemotherapies without increased toxicity are critically needed. One such strategy is cellular immunotherapy combined with well-tolerated chemotherapies. We found that the personalized cellular immunotherapy DC-CIK + S-1 was safe and resulted in favorable PFS and OS in treating APC. We propose to test this combination in randomized clinical trials.

which mediate non-MHC–restricted cytotoxicity. DC-CIK therapy has become the most widely used cellular immunotherapeutic, largely due to the rapid proliferation of this cell type *in vitro* as well as its strong antitumor activity against a broad spectrum of solid tumors (11, 12). Our previous study indicated that DC-CIK infusions activated cellular immune responses and when combined with chemotherapy, improved the clinical outcome of patients with advanced cancer (13). In the current study, we have prospectively investigated the clinical effects and safety of DC-CIK administered with S-1 for the treatment of patients with advanced pancreatic carcinoma and observed that the combination was associated with a longer progression-free and overall survival compared with either alone or best supportive care.

Patients and Methods

Study design

The study (ClinicalTrials.gov identifier, NCT01781520; https://register.clinicaltrials.gov/) was approved by the Regional Ethical Review Board for Capital Medical University Cancer Center. Patients were treated according to the Declaration of Helsinki's ethical principles for medical research involving human subjects. The trial was performed according to Good Clinical Practice guidelines. All patients provided an informed written consent prior to study entry. Patients were required to meet the following inclusion criteria: age $\geq$18 and $\leq$80 years; Eastern Cooperative Oncology Group performance status (ECOG-PS; ref. 14) of 0–2; histologically or cytologically confirmed unresectable, locally advanced, or metastatic adenocarcinoma of the pancreas. No prior immunotherapy or chemotherapy for metastatic or locally advanced disease was allowed. Patients were excluded if they had a concurrent malignancy other than pancreatic cancer, a serious, uncontrollable medical condition, or a psychiatric disorder that would limit ability to comply with study requirements. The purpose of this study was to establish the safety of the combination of DC-CIK with S-1. Consecutive patients with advanced pancreatic cancer were offered the study combination and those who chose only monotherapy, chemotherapy, or supportive care were enrolled into parallel control arms.

Generation and assessment of DC-CIKs

CIK cells were prepared as described in our previous studies (15, 16). Mononuclear cells were harvested from peripheral blood and expanded *in vitro* with IL2. For the induction of DC- CIKs, peripheral blood mononuclear cells were mobilized by G-CSF. Apheresis was performed using the COBE Spectra cell separator (COBE BCT) until reaching a threshold of $CD34^+$ cells of $\geq 4.5 \times 10^6$/kg. The apheresis product (25–50 mL) was cocultured with IL4, TNFα, and GM-CSF *in vitro* for 7 days to generate autologous DCs. After meeting lot release criteria, the cultured cells were infused intravenously over 20 minutes.

Treatment scheme

The treatment groups were as follows: DC-CIK plus S1, DC-CIK alone, S-1 alone, and best supportive care. The dose of S-1 was determined according to the body surface area as follows: <1.25 m^2, 40 mg; 1.25–<1.5 m^2, 50 mg; and $\geq$1.5 m^2, 60 mg, given twice daily after meals for 14 days followed by a 7-day rest. Cycles were repeated every 21 days. Treatment was continued until disease progression, unacceptable toxic effects, or the withdrawal of consent. In patients who received DC-CIK cell therapy, the DC-CIK cells were infused on days 15, 17, and 19 of 21-day cycles. A median of 7.8×10^9 CIK cells were infused every cycle.

Identification of somatic alterations in APC patients

Plasma samples were collected before initial treatment and three months after initiating the DC-CIK. Next-generation sequencing was performed on peripheral blood cell-free DNA (cfDNA) by a commercial vendor (Geneplus-Beijing Institute, Beijing, China). Targeted sequencing was performed in 28 plasma cell-free DNA (cfDNA), as well as 14 germline DNA. The target region is about 1.1 Mb which include coding exons and selected introns of 1,021 genes with known roles in cancer. About 10 Gb and 2 Gb sequencing data were generated for each cfDNA sample and gDNA sample, respectively. The average coverage of depth was 1,323-fold (706–2,094) for cfDNA samples. Somatic SNVs and InDels were detected using the Mutect 2.0 algorithm (https://software.broadinstitute.org/gatk/gatkdocs/current/org_broadinstitute_gatk_tools_walkers_cancer_m2_MuTect2.php). Somatic copy number alterations and structure variations were analyzed using local algorithms. In total, 93 SNV mutations and 15 InDels mutations were identified in cfDNA samples.

Target capture and next-generation sequencing

A total of 368 genes were selected from four sources: (i) known oncogenes and tumor suppressor genes; (ii) genes that are targets of agents approved by the FDA or have been assessed in clinical trials; (iii) genes implicated in major cancer-related signaling pathways; (iv) genes identified in the findings of the TCGA network which covers 12 cancer types. The target-capture region was 1.9 Mb in size and designed for all exons from 368 genes. Sequencing libraries were prepared from ctDNA using KAPA DNA Library Preparation Kits (Kapa Biosystems, Inc.), and gDNA sequencing libraries were prepared using the protocols recommended by the Illumina TruSeq DNA Library Preparation Kit. For samples close to the minimum input requirement, additional precapture PCR cycles were performed to generate sufficient PCR product for hybridization.

Follow-up

Following treatment, serum CEA, CA19-9, CA-153, and CA-125 were obtained and abdominal ultrasound was performed in all patients monthly. Abdominal contrast-enhanced CT scans or MRIs were performed every 3 months. Further

investigations were carried out when clinically indicated or when tumor progression was suspected. OS was defined as the period from the date of first treatment until death. Patients who did not experience an event were censored on the date of last contact. PFS was defined as the period from the date of first treatment until occurrence of an event (progressive disease, death, diagnosis of a second malignant neoplasm), whichever occurred first.

Analysis of the circulating immune response

Peripheral venous blood was obtained from each patient at various time points after DC-CIK infusion. Whole blood (100 μL) was incubated in the dark with primary antibody at 4°C for 15 minutes. Anti-CD3-FITC/anti-CD56-RPE (Dako), anti-CD3-FITC (fluorescein isothiocyanate), anti-CD4-RPE, anti-CD8-RPE, anti-CD45RO, and anti-CD4-FITC/anti-CD25-PE (BD Biosciences) were used. After hemolysis for 10 minutes, samples were centrifuged for 10 minutes at 1,500 rpm at room temperature, and then washed twice in PBS and subjected to flow cytometric analysis. Three-color flow cytometric analysis was performed to determine cell phenotypes using an FC500 (Beckman-Coulter), and CXP analysis software (Beckman-Coulter). Lymphocytes were gated by forward scatter versus side scatter. Analysis was set to collect 5,000 gated events.

Statistical analysis

Continuous variables were expressed as mean ± SD and compared using a two-tailed unpaired Student *t* test; categorical variables were compared using χ^2 or Fisher analysis. Life-table estimates of survival time were calculated according to the Kaplan and Meier methodology (17). The Greenwood formula was used for the SD. A Cox proportional hazards regression approach (18) was chosen for the evaluation of PFS and OS as the primary endpoint. Potential prognostic variables were analyzed both univariately with one factor taken at a time, and then in a multivariate model combining all factors. Results are reported as HRs and their 95% confidence intervals (CI). A HR > 1 indicated an elevated risk with respect to the reference category. A confidence interval which did not include the value 1 indicated statistical significance at the 5% level. All statistical evaluations were carried out using SPSS software (Statistical Package for the Social Science, version 15.0, SPSS Inc). A value of $P < 0.05$ was considered to be statistically significant in all the analyses.

Results

Patients' characteristics

Patients were enrolled at the Capital Medical University Cancer Center, Beijing Shijitan Hospital from June 1, 2013 to May 30, 2016. The 47 patients in this study were treated with either DC-CIK cell therapy alone ($n = 11$), S-1 therapy alone ($n = 4$), DC-CIK combined with S-1 therapy ($n = 25$), or best supportive care ($n = 7$). Characteristics of all patients are detailed in Table 1. There were no significant differences in relevant baseline characteristics between the treatment groups.

Treatment toxicity

None of the enrolled patients who received DC-CIK and S-1 failed to complete a full course of therapy or were ruled out because of side effects. No severe adverse effects (grade 3 or grade 4) were associated with D-CIK and S-1 therapy, and common grade I–II toxicities consisted of transient fever (17.5%), chills (15%), fatigue (22.5%), headache (7.5%), and anemia (7.5%).

Clinical outcomes

The disease control rate were 0%, 45%, 50% and 80% in Supportive care, DC-CIK, CT, and DC-CIK plus S-1 groups, respectively. There were significant differences among these groups ($P = 0.002$; Table 1).

Survival analysis of patients with APC

For all the patients, the median PFS was 99 days (95% CI, 43–116 days), and the median OS was 143 days (95% CI, 65–268 days). The 6-month PFS and OS rates were 20.9% and 38.7%, respectively.

Table 1. Demographics and baseline characteristics of patients

Variable	Supportive care group	DC-CIK group	CT group	DC-CIK combined with CT group	*P*
Case, *n*	7	11	4	25	
Age	70.1 ± 7.6	68.4 ± 4.6	71.5 ± 3.7	70.4 ± 6.1	0.325
Sex					0.846
Female	4	7	2	12	
Male	3	4	2	13	
ECOG-PS					0.783
1	3	6	1	11	
2	4	5	3	14	
TNM staging					0.459
III	1	3	1	2	
IV	6	8	3	23	
Site of metastasis					0.571
Liver	2	3	2	12	
Lung	2	2	1	4	
Peritoneum	1	5	1	10	
Bone	3	1	1	4	
Other	0	2	1	4	
Disease control					0.002
Stable	0	5	2	20	
Progressive	7	6	2	5	

Abbreviation: CT, chemotherapy.

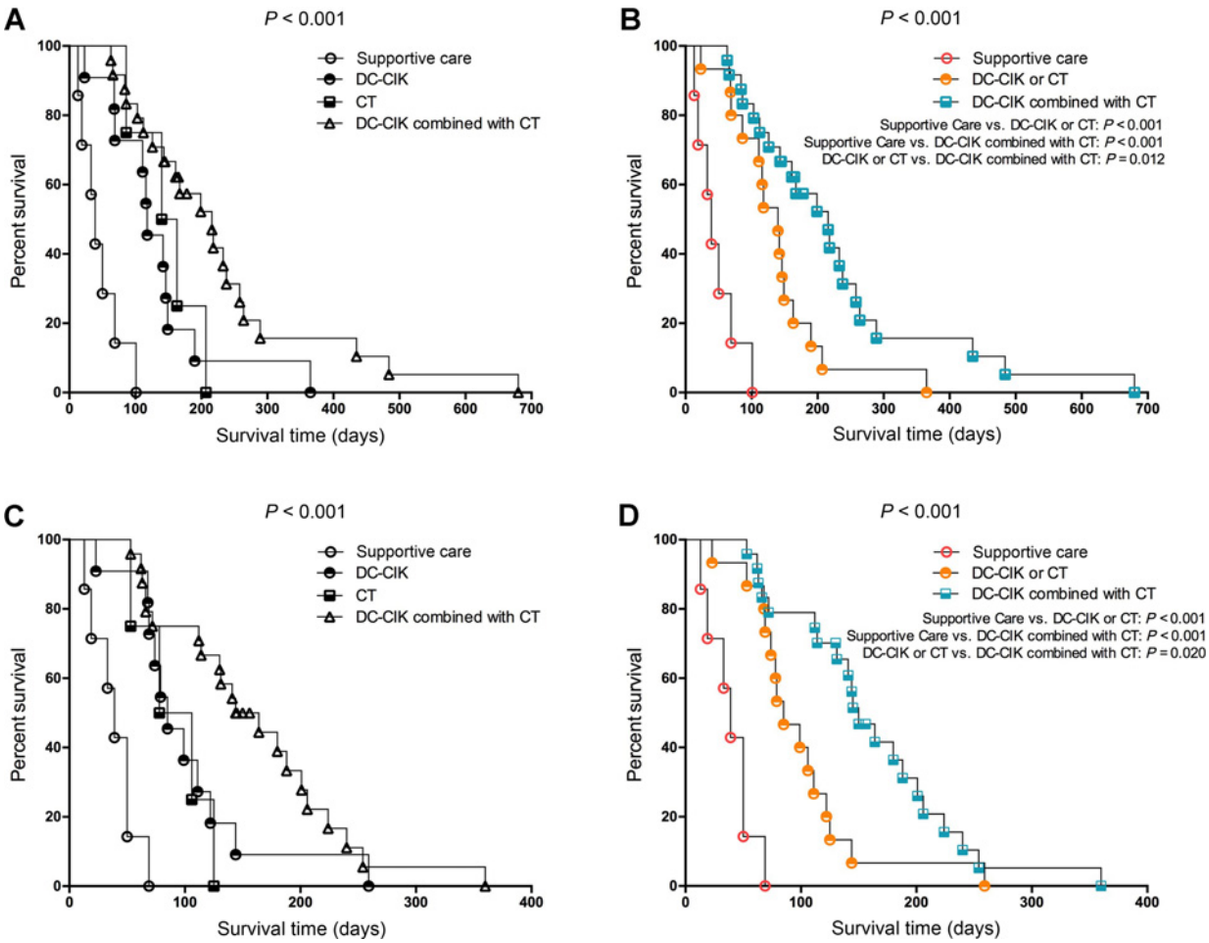

Figure 1.
Overall survival (OS; **A** and **B**) and progression-free survival (PFS; **C** and **D**) for the different treatment groups.

The 6-month PFS for DC-CIK plus CT (41.6%; 95% CI, 27.5%–56.2%) was significantly higher than that in the group of patients who received DC-CIK alone (9.09%; 95% CI, 2.6%–12.7%), CT (0%), and the group with supportive care alone (0%; $P < 0.001$, Fig. 1A and B). Meanwhile, the 6-month OS rate of DC-CIK combined with CT (62.2%; 95% CI, 48.7%–78.4%) was significantly higher than that in the DC-CIK (18.2%; 95% CI, 10.7%–25.4%), CT (25%; 95% CI, 12.5%–37.5%), and the group with no therapy (0%; $P < 0.001$, Fig. 1C and D).

Among all patients who received 2 or more cycles of DC-CIK, there was a higher median PFS (143 vs. 92 days for those who received DC-CIK for ≥2 cycles versus 1 cycle) and 6-month PFS rate (37.6% vs. 19.2%; $P = 0.030$, Fig. 2A). Moreover, patients who received 2 or more cycles of DC-CIK had a higher median OS (195 vs. 117 days for the group of DC-CIK ≥2 cycles vs. 1 cycle) and 6-month OS rate from 19.2% to 56.3% ($P = 0.011$, Fig. 2B). We performed stratified analysis in the group of patients who received DC-CIK plus S-1. We demonstrated that patients who received DC-CIK for ≥2 cycles had a significantly longer PFS (Fig. 2C) and OS (Fig. 2D) compared with patients who received 1 cycle of DC-CIK cell therapy.

Predictors associated with clinical outcomes

Cox proportional hazards models were then used to quantify the prognostic significance of risk factors after multivariable adjustment. A multivariable analysis was performed to assess the factors that demonstrated significant effects as in univariate analysis. After adjusting for competing risk factors, DC-CIK combined with CT and receipt of 2 or more cycles of DC-CIK treatment remained independent predictors of disease free and overall survival ((HR: 0.583; 95% CI: 0.342–0.877, $P = 0.003$ and HR: 0.458; 95% CI: 0.335–0.766, $P = 0.001$). Number of sites of metastases >1 was associated with adverse prognosis in patients with APC (HR: 1.364; 95% CI: 1.158–1.843, $P = 0.041$ and HR: 1.441; 95% CI: 1.114–1.935, $P = 0.031$). The details are shown in Table 2.

Changing trend of tumor biomarkers among different groups

CEA and CA-199 levels decreased in the greatest percentage of patients in the DC-CIK plus S1 group ($P = 0.034$ and $P = 0.025$,

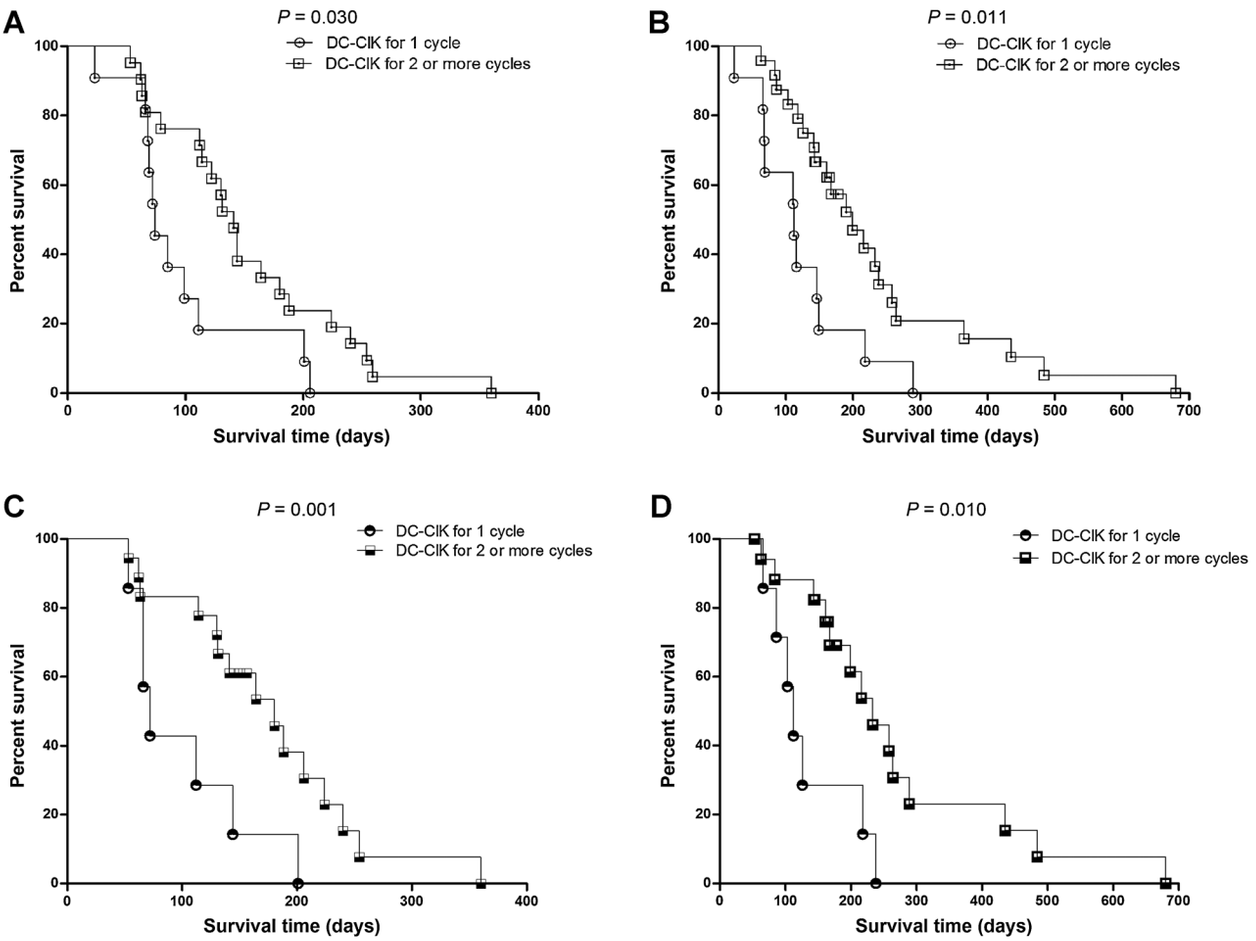

Figure 2.

Progression free survival and overall survival for patients who received DC-CIK cell therapy divided into 1 cycle and 2 or more cycles among different groups. Progression free survival (**A**) and overall survival (**B**) for patients in DC-CIK plus S1 group and progression free survival (**C**) and overall survival (**D**) for patients in DC-CIK group divided into 1 cycle and 2 or more cycles.

respectively, Fig. 3A and C). The CA-125 and CA-153 showed no significant differences (Fig. 3B and D).

Phenotypic analysis of peripheral blood immune cells

Phenotypic analysis of peripheral blood mononuclear cells before the treatment and at the end of the first cycle of therapy demonstrated that the $CD3^+$, $CD3^+/CD4^+$ and $CD8^+/CD28^+$ T-cell subsets were increased after DC-CIK cell therapy ($P < 0.05$), while the $CD3^+/CD8^+$, $CD3^+/CD16^+/CD56^+$ and $CD4^+/CD25^+$ cell subsets were significantly decreased after DC-CIK cell therapy ($P < 0.05$; Fig. 4).

Genomic profile identified of cfDNA in patients with APC

We identified copy number variants (CNV) in 14 of 25 (56%) in plasma samples by analyzing the sequencing data in the patients who received treatment with DC-CIK (Supplementary Table S1). To determine whether a decrease in cfDNA could reflect treatment response to DC-CIK infusions, we analyzed

Table 2. Multivariable Cox proportional hazard regression analysis of patients' demographic and clinical characteristics and survival

	PFS		OS	
Variables	**HR (95% CI)**	***P***	**HR (95% CI)**	***P***
DC-CIK treatments ≥2 cycles	0.462 (0.314–0.806)	0.025	0.759 (0.684–1.171)	0.073
ECOG-PS: 2	1.071 (0.604–1.352)	0.641	1.135 (0.871–1.371)	0.264
TNM staging: IV	1.048 (0.827–1.282)	0.886	1.102 (0.923–1.215)	0.697
No. of metastases > 1	1.364 (1.158–1.843)	0.041	1.441 (1.114–1.935)	0.031
Therapeutic modalities: DC-CIK combined CT	0.583 (0.342–0.877)	0.003	0.458 (0.335–0.766)	0.001

Abbreviation: CT, chemotherapy.

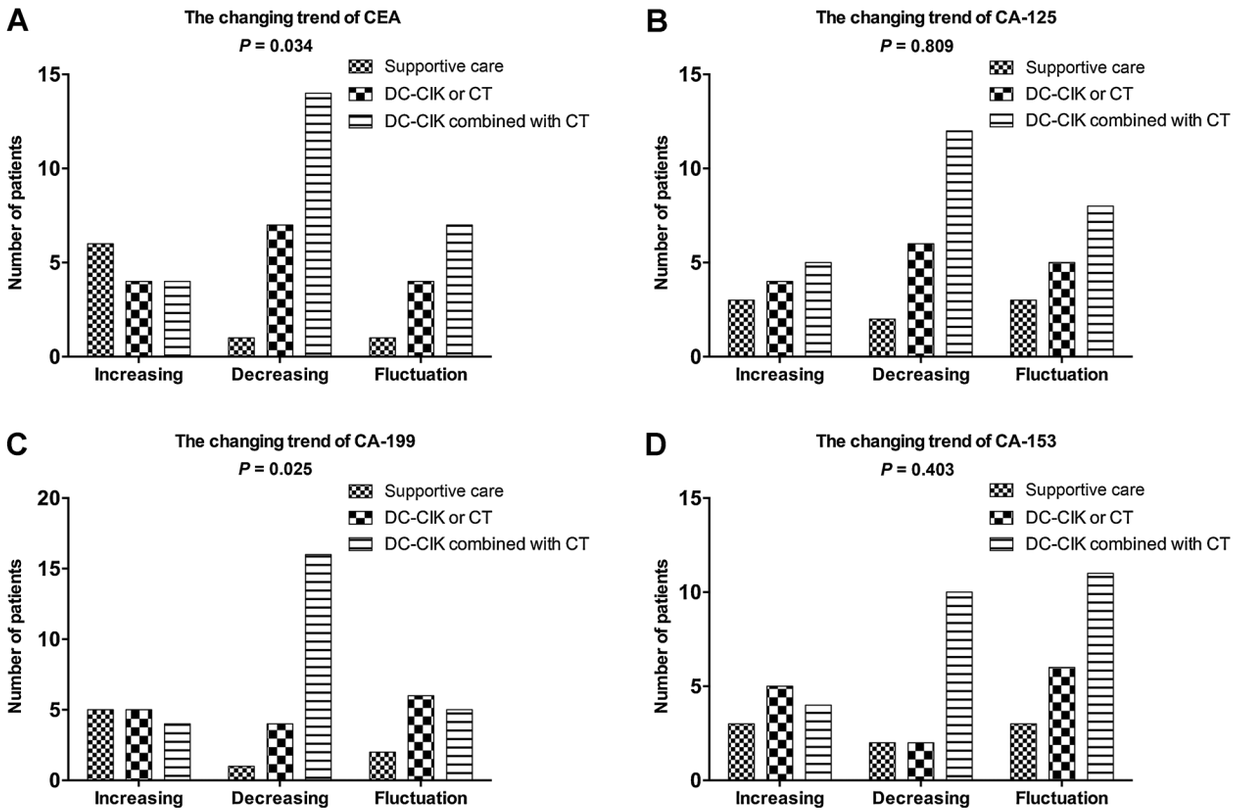

Figure 3.
Changing trend of tumor biomarkers among different groups.

tumor burden in serial blood samples of these 14 patients. Four patients had tumor burden decrease after treatment with DC-CIK infusions and had a relatively superior prognosis compared with others (Supplementary Fig. S1). These data provide the basis for further research on selection of patients who are suitable for receiving DC-CIK.

Discussion

Poor outcomes for pancreatic carcinoma remain a challenge, despite the application of multiagent chemotherapy. Similarly, immunotherapy with vaccines and immune checkpoint blockade has had limited efficacy, in part, due to inadequate effector cell responses (19). In this study, we combined the oral chemotherapy S-1, which has demonstrated antitumor activity in pancreatic cancer (7) along with DC-CIK immunotherapy, which allows delivery of both DCs with potent capacity for antigen presentation and induction of adaptive immune responses and NK-T-like cells with innate cytotoxic capacity. Although previously there had been concern that chemotherapy would inhibit the immune response, preclinical and clinical data now indicate the feasibility, safety, and immunogenicity of combinations of chemotherapy and immunotherapy in gastrointestinal malignancies including pancreatic cancer (20–26). Proposed mechanisms include chemotherapeutic control of tumor growth until immune cells achieve adequate activation, release of tumor antigens for cross presentation by dendritic cells, upregulated expression of tumor-associated antigens, decreases in regulatory T cells or immune inhibitory cytokines such as TGFβ, and enhancement of intratumoral accumulation of immune effectors. We observed that combining DC-CIK and S-1 resulted in modulation of the circulating immune effectors and lengthened survival compared with the DC-CIK or chemotherapy alone or best supportive care.

CA199, CA125, CA153, and CEA are routinely used in clinical practice to determine prognosis and monitor therapeutic responses in gastroenterologic cancers. Among these, the most common and best-identified marker for pancreatic cancer is CA19-9 (27). Previous studies also showed that serum CA125 and CEA are important tumor biomarkers for the early diagnosis of pancreatic cancer (28, 29). In this study, our results showed that CEA and CA-199 levels decreased in the greatest percentage of patients in the DC-CIK plus S1 group, and was associated with better outcome compared with other groups.

Because tissue available to analyze intratumoral immune responses is often limiting in pancreatic cancer, we analyzed changes in T-cell subsets in the peripheral blood before and after the DC-CIK therapy. The $CD3^+$, $CD3^+/CD4^+$ and $CD8^+/CD28^+$ T-cell subsets were increased after DC-CIK cell therapy, while the $CD3^+/CD8^+$, $CD3^+/CD16^+/CD56^+$ and $CD4^+/CD25^+$ cell subsets were significantly decreased. CD28 is involved as the receptor for the second signal of T-cell activation and is a marker for an effector memory population of T cells following adoptive

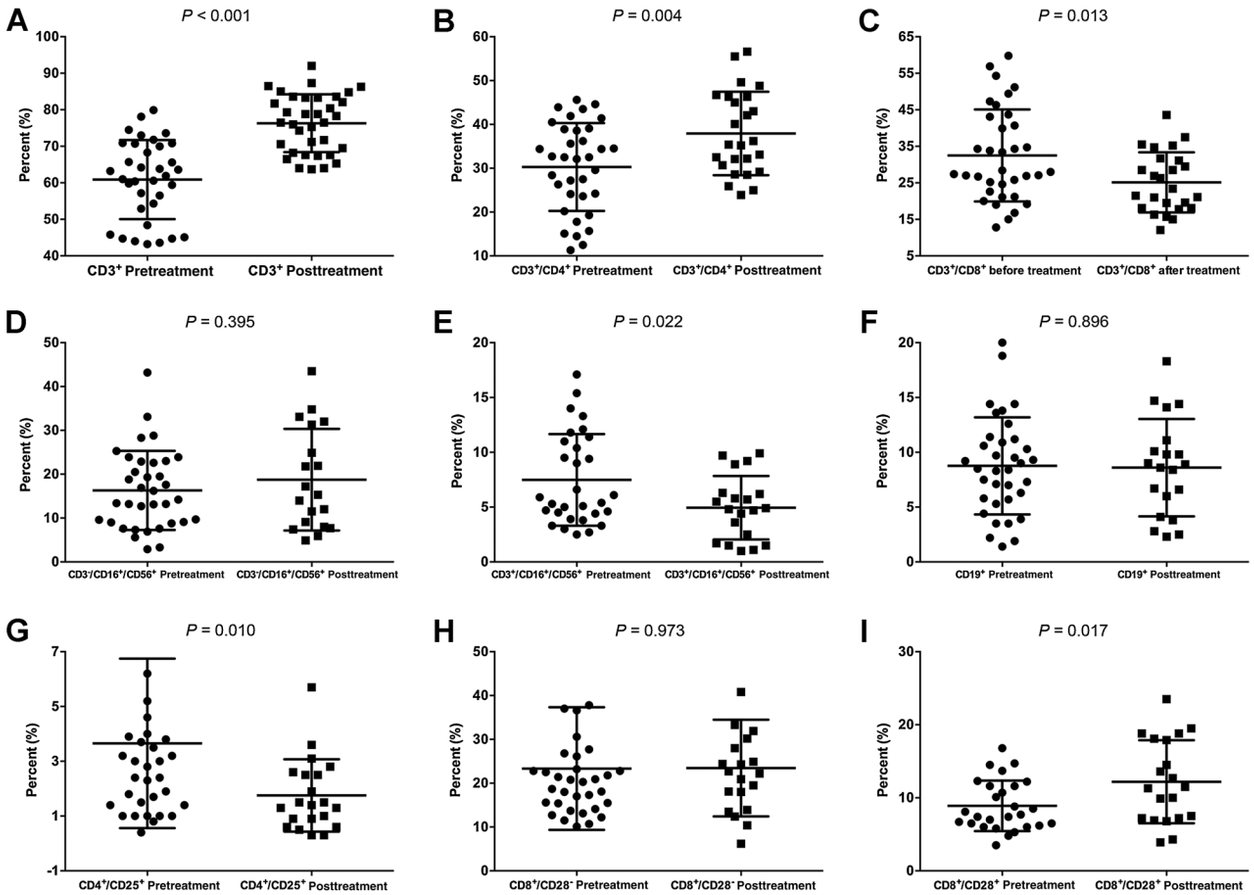

Figure 4.
Peripheral blood T-cell phenotype measurements via cytometry before and after the first cycle of DC-CIK cell therapy.

transfer (30). Interestingly, the $CD4^+CD25^+$ which may contain a population of regulatory T cells, was decreased after adoptive transfer of DC-CIK. We have recently demonstrated that DC-CIK immunotherapy could enhance the clonality based on T-cell receptor immune repertoire analysis (31). Therefore, the combination of S-1 with DC-CIK favorably modulates the immune milieu of the host.

Currently, there is considerable interest in identifying biomarkers that predict immunotherapy response. Bailey and colleagues reported an immunogenic subtype of APC through genomic analyses to select patients who might respond to immunotherapy (32). The immunogenic subtype included expression profiles related to infiltrating B and T cells, and upregulation of CTLA4 and PD1. In our study, we detected cfDNA in 14 patients before and after treatment with DC-CIK and considered that this may serve as a surrogate for tumor burden (33). To our knowledge, this was the first study to utilize cfDNA mutation frequency to evaluate the immunotherapy response and as an alternative measurement to measure tumor burden, particularly relevant with cancers such as APC for which tissue samples are often limiting.

Although the clinical activity demonstrated in this study is promising, one potential criticism is that there was no randomization and the results could reflect different patient baseline characteristics; however, it should be noted that, while not statistically different, there were a greater proportion of patients with stage IV and liver metastases in S1 plus DC-CIK immunotherapy group. Although there was a lower percentage of ECOG-PS 2 patients in the DC-CIK group, this was not found to be a prognostic factor on multivariate analysis. We believe that the favorable survival of patients treated with combined chemo/immunotherapy warrants further testing in a randomized trial using molecular subtype biomarkers to choose patients for enrolment.

Disclosure of Potential Conflicts of Interest

M.A. Morse reports receiving speakers bureau honoraria from Taiho. No potential conflicts of interest were disclosed by the other authors.

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Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): N. Jiang, G. Qiao, L. Zhou, Y. Song, F. Chen, L. Huang

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): G. Qiao, X. Yi, Y. Guan, J. Song

Writing, review, and/or revision of the manuscript: N. Jiang, G. Qiao, X.-L. Wang, M.A. Morse, W.R. Gwin, X.-N. Zhou, A. Hobeika, X. Xia, J. Ren, H.K. Lyerly
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): N. Jiang, X.-L. Wang, X.-N. Zhou, L. Huang, J. Ren, H.K. Lyerly
Study supervision: N. Jiang, G. Qiao, J. Ren

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