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Autologous Dendritic Cell-Cytokine Induced Killer Cell Immunotherapy Combined with S-1 Plus Cisplatin in Patients with Advanced Gastric Cancer: A Prospective Study

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Abstract

Purpose: We have assessed the combination of DC–CIK with S-1 plus cisplatin chemotherapy in advanced gastric cancer (AGC) and the role of mutational analysis of circulating tumor DNA (ctDNA) and T-cell receptor (TCR) repertoire in predicting clinical outcomes.

Patients and Methods: Consecutive patients ($n = 63$) with AGC were allocated to treatment with S-1 alone, S-1 plus cisplatin, DC–CIK combined with S-1 or DC–CIK combined with S-1 plus cisplatin. The primary endpoints were progression-free survival (PFS) and overall survival (OS) at 1 year; the secondary endpoints were disease control rate and analysis of ctDNA and TCR repertoire.

Results: The DC–CIK infusions were well tolerated with no serious adverse events. The disease control rates (CR^+PR^+SD) were 5.6%, 33.3%, 47.1%, and 76.9% in the S-1 alone, the S-1 plus cisplatin, DC–CIK combined with S-1 and DC–CIK combined with the S-1 plus cisplatin groups, respectively ($P = 0.001$). After adjusting for competing risk factors, treatment with DC-CIK combined with S-1 plus cisplatin was confirmed to be an independent predictor of PFS and OS ($P = 0.001$). A decrease in the frequency and number of mutations in ctDNA was observed in 19 patients (63.3%) following the DC–CIK infusions. Decreased ctDNA mutational frequency and restored TCR repertoire were associated with improved PFS and OS ($P = 0.001$).

Conclusions: DC–CIK combined with S-1 plus cisplatin provided a favorable PFS and OS in patients with AGC and the combination therapy was safe with tolerable toxicities. Clinical efficacy correlated with decreases in ctDNA mutational profiles and restored TCR repertoire.

Introduction

Gastric cancer (GC), the third leading cause of cancer-related mortality worldwide with more than 800,000 deaths annually, has its greatest incidence in East Asia (1). Unfortunately, the majority of newly diagnosed cases are identified in the advanced stage (2) for which outcomes are extremely poor marked by a median survival ranging from 3 to 5 months with best supportive care (3, 4). Several multiagent chemotherapy regimens have demonstrated modest survival benefits in the first line treatment (4). S-1 (5), an oral combination of the fluoropyrimidine tegafur, the DPD-inhibitor gimeracil, and oteracil potassium, intended to reduce gastrointestinal toxicity of fluorouracil, is commonly used in Asia for management of gastrointestinal malignancies including GC (6, 7). A network meta-analysis reported that S-1 regimens were more effective than fluorouracil in advanced GC (8). Furthermore, a recent meta-analysis has concluded that compared with capecitabine-based therapy, S-1–based chemotherapy has non-inferior antitumor efficacy and a better safety profile in advanced GC (9). The randomized phase III SPIRITS trial showed the superiority of S-1 plus cisplatin combination therapy to S-1 monotherapy in the first-line treatment of Japanese patients with AGC (10–12), solidifying this combination as the standard first line chemotherapy combination in Asian centers.

Recently, cancer immunotherapy has demonstrated encouraging activity in GC. In the double-blinded, randomized, phase III trial of nivolumab as salvage treatment after second or later-line chemotherapy for advanced gastric or gastro-esophageal junction cancer (13), the median OS was 5.32 months with nivolumab versus 4.14 months with placebo (hazard ratio, 0.63, $P < 0.0001$) and the overall response rate was 11.2% with nivolumab versus 0% with placebo ($P < 0.0001$). Pembrolizumab is approved by the FDA for PD-L1–positive recurrent or metastatic adenocarcinoma of the stomach or gastro-esophageal junction with disease progression on or after two or more prior lines of therapy, including fluoropyrimidine- and platinum-containing chemotherapy and if appropriate, HER2/neu-targeted therapy based on a 22%

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Translational Relevance

We did a prospective study of DC–CIK combined with chemotherapy for patients with advanced gastric cancer. We have found that DC–CIK combined with S-1 plus cisplatin could generate favorable PFS and OS. Clinical benefit was predicted by changes in ctDNA mutational profiles and TCR repertoire.

response rate in the KEYNOTE-012 study (14). Moreover, recently studies showed that microsatellite instability(MSI) regulated immune response and it was one of the most promising targets for GC immunotherapy (15, 16). These results, albeit modest, do demonstrate the immunologic sensitivity of AGC.

It is hypothesized that one explanation for modest efficacy of the anti–PD-1/anti–PD-L1 therapies is the limited T-cell infiltration into some GCs (17). One potential solution is to adoptively deliver *ex vivo* activated cellular products such as dendritic, NK, or T cells (18). Dendritic cells (DC), are potent stimulators of tumor-specific T-cell responses. Cytokine-induced killer (CIK) cells are *ex vivo*-expanded T lymphocytes with a natural killer/T-cell phenotype expressing both CD56 and CD3, which mediate non-MHC restricted cytotoxicity. DC–CIK therapy has become widely used adoptive cellular immunotherapy (19), largely due to ease of generation, rapid expansion *ex vivo*, strong antitumor activity against a broad spectrum of solid tumors, and record of clinical safety. Our previous studies have demonstrated that DC-CIK infusions activated cellular immune responses and when combined with chemotherapy, improved the clinical outcome of patients with advanced cancers (20). In a prospective clinical trial, we observed synergistic anti-cancer activity for the combination of DC-CIK and S-1 in patients with advanced pancreatic cancer (21).

Circulating tumor DNA(ctDNA) and T-cell receptor (TCR) sequencing are emerging approaches for identifying corresponding mutations from the primary tumor and allowing characterization of the T-cell repertoire, respectively, in an increasing number of studies (22, 23). Plasma ctDNA species are identifiable by the presence of pathognomonic or previously characterized molecular alterations in corresponding tumor tissue. Recent advances in our understanding of the biologic properties and clinical associations of ctDNA, as well as the data analytic platforms for its detection, have provided evidence that this class of biomarker may also enable a level of sensitivity suitable for noninvasive tumor monitoring (24) The somatic alterations detected in ctDNA are directly derived from an individual tumor. Somatic DNA alterations therefore can be thought to define the presence and level of ctDNA. The reactivity of the TCRs expressed by tumor-infiltrating lymphocytes (TIL) determines their capacity to interact with tumor antigens presented on antigen-presenting cells (APC). Accordingly, ctDNA mutations can be used to identify potentially actionable changes affecting driver genes as well as providing personalized biomarkers that can be used to detect residual disease or monitor tumor levels during therapy, and the TCR repertoire has been reported to be associated with response to immune checkpoint blockade and survival in patients with cancer (25, 26). In light of our previous work suggesting a decreasing proportion of ctDNA mutations correlated with the treatment of ACT and patients' outcomes, and considering the role of the T-cell repertoire in the antitumor response, we next sought to assess the relationship between the TCR repertoire and prognosis and ctDNA mutations in different patients.

In the present study, we evaluated the combination of DC/CIK cell immunotherapy with chemotherapy in AGC. Furthermore, we analyzed ctDNA mutational profiles and TCR repertoire for their association with the efficacy of DC–CIK cell immunotherapy.

Patients and Methods

Study design and participants

This was a prospective study carried out in a single center in Beijing, China. Participants were age 18 to <80 years with advanced, unresectable or metastatic gastric adenocarcinoma. In addition, patients had to meet the following criteria: Eastern Cooperative Oncology Group performance status (ECOG-PS; Ref. 27) of 0–2; adequate organ function; and an expected survival of at least 3 months. Adequate organ functions as established by tests performed within the 14 d before enrolment as follows: leucocyte count of $4.0–12.0 \times 10^9$/L; neutrophil count $>2.0 \times 10^9$/L; platelets count $>100 \times 10^9$/L; hemoglobin >80 g/L; serum aspartate aminotransferase and alanine aminotransferase <100 U/L; serum bilirubin <1.50 mg/dL; serum creatinine <upper limit of normal; and creatinine clearance >50 mL/min. When patients were consent with enrollment into the study, the previous chemotherapeutic regimens and all physical and patients' reported toxicities and intolerance should be recorded and analyzed. Patients could have no serious comorbidities precluding physically functional disorders and psychologically rejection or reluctant to the chemotherapeutic drug(s) as well when their previous experiences were perilously considered. Patients were ineligible if they had a concurrent malignancy other than AGC, a serious, uncontrolled medical condition, or a psychiatric disorder that would limit ability to comply with study requirements. The AJCC 7^{th} TNM staging system was used to evaluate the staging of patients with AGC (28).

In this study we have continuously validated the patient allocation design termed as the prospective patient's preference-based study (PPPS) in which the prospective assignment of patients with similar demographic characteristics to treatment cohorts is based on the patients' preference after complete communications and understandings of each possible accessible therapeutic option by the investigators, as we described in our previous studies (21, 29). Both the research documentation and progress were approved and supervised by institutional review board (IRB). The treatment decision was made by both peer-reviewed physicians and based on the well-communicated and outreached discussions with the patients and patients' family if applicable. We should state that such PPPS model might be executed more prevalent due to more and more precise medicine in oncology has been required. There was no any both extra toxicities and extra medical expenses occurrence. All the experimental relevant and sample analyses were covered by research funding in this study to protect each individual as audited by IRB. The study (ClinicalTrials.gov identifier, NCT01783951; https://register.clinicaltrials.gov/) was approved by the Regional Ethical Review Boards for Capital Medical University Cancer Center and all patients gave written consent for participation in the study. The study was conducted in accordance with the guideline of Declaration of Helsinki.

Generation of DC–CIKs

CIK cells were prepared as described in our previous studies (30, 31). Briefly, mononuclear cells were harvested from peripheral blood and expanded *in vitro*. For the induction of DC–CIKs, mobilization of PBMC was performed GM-CSF 5 mcg/kg sq per day (Chugai Pharm Co. Ltd.) to patients until the level of mononuclear cells reached 1.5×10^9/L. Then, PBMCs were separated by a COBE Spectra cell separator (COBE BCT) until $CD34^+$ reaching $\geq 4.5 \times 10^6$/kg. Then, 40 mL of the apheresis product was cocultured for 7 d with IL-4 (1,000 U/mL; R&D Systems, Inc.), TNF-α (20 ng/mL; R&D Systems, Inc.) and GM-CSF (800 U/mL; Amoytop Biotech Co., Ltd.) *in vitro* to generate autologous DCs. Mononuclear cells were separated by gradient centrifugation and activated in vitro with the recombinant cytokines IL-2 at 1,000 U/mL (Boehringer Mannheim, Germany), IFNγ at 1,000 U/mL (Boehringer Mannheim, Germany) and CD3 antibody at 1.7 mL/mL (Boehringer Mannheim, Germany) for 7 to 10 days. The phenotypes of DCs (CD80, CD86, HLA-DR, CD1a, and CD11c) and CIKs (CD3 and CD56) were characterized by flow cytometry. The proportion of $CD80^+$ plus $CD86^+$ cells reached greater than 80% among the cultured cells in the autologous DC-specific cultures. The cultured autologous DCs were then mixed with cultured CIKs at a proportion of 1:100, and then DC–CIK were harvested for intravenous administration to patients.

Pretreatment evaluation

Medical history and physical findings were documented in each patient. Each patient also had an ECG, computed tomography of the abdomen and pelvis (and thorax, if needed), serum chemistry and CBC, and urine analysis.

Patient enrollment

Patients were enrolled prospectively into one of four treatment groups: S-1 alone, S-1 plus cisplatin, DC–CIK combined with S-1 and DC–CIK combined with the S-1 plus cisplatin. We used an allocation strategy as in our previous study (21) whereby the different study arms were explained and patients chose their preferred arm. As we noted, we were encouraged to continuously explore the clinical efficacy of prospective patient's preference-based study (PPPS) in which patient protection-based enrollment was throughout as core principle. For the choice of the chemo regimens, S-1 plus cisplatin or S-1 alone, the physicians seriously discussed and considered with the patient 's previous chemo/therapeutic exposures and the experienced clinical outcomes and toxicities of multiple chemo regimens. Meanwhile, being afraid of potential severe side effects in digestive tract and leukopenia of cisplatin, a few patients refused to apply IV chemotherapy and therefore were allocated in S-1 alone subgroup.

Because the primary endpoints were progression-free survival (PFS) and overall survival (OS) at 1 year notably to compare the roles of supplemental DC/CIK immunotherapy into the standard chemotherapy, the comparative molecular profiles studies, including ctDNA mutations and TCR repertoire were incorporated as exploratory measurements to testify whether those genetic alterations could be valuable as the prognostic markers in the clinical practice. As we may be aware of quite limited OS among the AGC from the published literatures, therefore we have determined those endpoints. Although the present non-randomized but prospective study adapted the similar design of previous combined chemotherapy, the implementation of DC/CIK immunotherapy associated toxicity should also be alerted. Therefore, both disease control rate and toxicity were also recorded as the secondary endpoints. In addition, because no patients' tumors were HER2-positive by IHC, transtuzumab was not administered to any patients.

Procedures

The S-1 and cisplatin were obtained from commercial vendors. The dose of S-1 was determined according to the body surface area as follows: <1.25 m^2, 40 mg; 1.25 to <1.5 m^2, 50 mg; and ≥ 1.5 m^2, 60 mg, given twice daily after meals for 14 days followed by 7 days off. Cisplatin was administered at 75 mg/m^2 intravenously over 1 to 3 hours every 21 days. Cycles were repeated every 21 days. Treatment was continued until disease progression, unacceptable toxic effects, or the withdrawal of consent. One cycle of DC/CIK immunotherapy included three cellular infusions after chemotherapy. Patients received DC–CIK cell therapy at days 15, 17, and 19 for the first and repeatedly the second cycle was given after the second chemotherapy administrated. All patients in DC–CIK combined with chemotherapy arms of the study received 2 cycles of DC–CIK cell infusions. A median of 1.27×10^7 DC and 2.8×10^9 CIK cells were infused in the first cycle.

Identification of somatic alterations in patients with AGC

Plasma samples were collected before initial treatment and three months after initiating the DC–CIK. Next-generation sequencing was performed on peripheral blood ctDNA by a commercial vendor (Geneplus-Beijing Institute, Beijing). Targeted sequencing was performed in 60 plasma ctDNA, as well as 30 germ line DNA samples. The target region is about 1.1 Mb, which include coding exons and selected introns of 1021 genes. A total of 1,021 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. Sequencing libraries were prepared from ctDNA using KAPA DNA Library Preparation Kits (Kapa Biosystems, Inc.), and genomic DNA (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 pre-capture PCR cycles were performed to generate sufficient PCR product for hybridization. Libraries were hybridized to custom-designed biotinylated oligonucleotide probes (Integrated DNA Technology) covering target region sequence. DNA sequencing was carried out with the HiSeq3000 Sequencing System (Illumina).

Somatic SNVs and InDels were detected using the Mutect 2.0 algorithm (https://software.broadinstitute.org/gatk/documentation/tooldocs/current/) Somatic copy-number alterations and structure variations were analyzed using local algorithms.

TCR sequencing

DNA was extracted from *ex vivo* expanded T cells using a Qiagen DNA FFPE kit, DNA blood kit, or DNA blood mini kit (Qiagen). TCR Vb CDR3 sequencing was performed using the survey (cultured cells) or deep (PBMC) resolution Immunoseq platforms. Bioinformatic and biostatistical analyses of productive clones were performed to assess the dynamics of expanded T cells. The *ex vivo* TCR repertoire on day 15 of the expansion was compared to the initial TCR repertoire. The clonality of expanded T-cell was

used to evaluate the diversity of TCR V-beta CDR3 sequences for AGC patients.

Response and adverse event assessments

Clinical and laboratory examinations were carried out within 7 days before enrollment and each cycle of chemotherapy afterward. Tumor measurement was conducted on the basis of computed tomographic scans, within 15 days before enrollment and every 3 months in the admission. Discontinuation of therapy occurred in the event of progression of disease, patient refusal, unacceptable toxicity, or death. 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 an occurrence of an event (progressive disease, death, diagnosis of a second malignant neoplasm), whichever occurred first. Disease control rate (DCR) was defined as the percentage of patients with advanced or metastatic cancer who achieved complete response, partial response and/or stable disease, based on the revised RECIST guideline (version 1.1; ref. 32) and the immune-related response criteria (33). DCR was confirmed by repeat assessments performed 3 months after the criteria for response were first met. Adverse events were assessed according to the National Cancer Institute's Common Terminology Criteria for Adverse Events version 4.0 (NCI-CTCAE v4.0).

Phenotypic analysis of peripheral blood immune cells

Peripheral venous blood was obtained from each patient both before and after the treatment of 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. Three-color flow cytometric analysis was performed to determine cell phenotypes using an FC500 (Beckman-Coulter), and CXP analysis software (Beckman-Coulter), as described in our previous study (21).

Statistical analysis

Continuous variables were expressed as mean ± SD (standard deviation) 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 (34). The Greenwood formula was used for the standard deviation. A Cox proportional hazards regression approach (35) was chosen for the evaluation of DFS and OS as the primary end-point. 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 hazard ratios (HR) and their 95% confidence intervals (CI). All statistical evaluations were carried out using SPSS software (Statistical Package for the Social Science, version 15.0, SPSS Inc.) and GraphPad Prism 5 (Version 5.01, GraphPad Software, Inc.). A value of $P < 0.05$ was considered to be statistically significant in all the analyses (21, 29).

Results

Patients' characteristics

Patients were enrolled at the Capital Medical University Cancer Center, Beijing Shijitan Hospital from December 1, 2013 to June 30, 2016. The 63 patients included in this study were allocated into four groups: DC-CIK combined with S-1 plus cisplatin ($n = 13$), S-1 plus cisplatin ($n = 15$), DC-CIK combined with S-1 ($n = 17$) and S-1 alone ($n = 18$). Characteristics of all patients are detailed in Table 1. There were no significant differences in relevant baseline characteristics between these treatment groups.

Clinical outcomes

In this study, the DCRs were 5.6%, 33.3%, 47.1%, and 76.9% in S-1 alone, S-1 plus cisplatin, DC-CIK combined with S-1 and DC-CIK combined with S-1 plus cisplatin groups, respectively. There were significant differences among these groups ($P = 0.001$; Table 2).

Survival analysis of patients with AGC

Median follow-up was 417 days [95% confidence interval (CI), 270–769]. For all the patients, the median PFS was 176 days (95% CI, 74–228 days), and the median OS was 400 days (95% CI, 203–568 days). The 1-year PFS and OS rates were 30.9% and 51.5%, respectively. There were significant differences in PFS and OS among the four groups ($P < 0.001$, Fig. 1A and B); specifically, the 1-year OS rate for DC-CIK combined with S-1 plus cisplatin (87.5%) was significantly higher than that in the groups of patients who received DC-CIK combined with S-1 (59.9%, $P = 0.041$), S-1 plus cisplatin (53.7%, $P = 0.042$), and S-1 alone (22.2%; $P < 0.001$; Fig. 1A). Also, the 1-year PFS rate for DC-CIK combined with S-1 plus cisplatin (76.9%) was significantly higher than that in the groups of patients who received S-1 plus cisplatin (31.9%, $P = 0.031$) and S-1 alone (5.6%, $P < 0.001$; Fig. 1B). There was a non-significant difference shown between the group of DC–CIK combined with S-1 plus cisplatin and DC–CIK combined with S-1 (33.9%) on PFS ($P = 0.067$; Fig. 1B). There is a trend for better PFS and a real benefit in OS within the groups DC–CIK combined with S-1 plus cisplatin versus DC-CIK combined with S-1, and compared with the other groups with chemo alone. Moreover, the 1-year PFS and OS rates of DC-CIK combined with S-1 and S-1 plus cisplatin were significantly higher than that in the S-1 alone, respectively (Fig. 1A). No significant difference was shown between the group of DC–CIK combined with S-1 and S-1 plus cisplatin on both OS and PFS (Fig. 1A and B). In a stratified analysis according to the different treatment modes, death events occurred in 8 (28.6%) of the 28 patients assigned S-1 plus cisplatin, and in 23 (65.7%) of the 35 patients assigned S-1 alone. There were significant differences in both PFS and OS between these two groups ($P < 0.05$, Fig. 2A and B). Death events occurred in 8 (26.7%) of the 30 patients assigned to DC–CIK, and in 23 (69.7%) of the 33 patients assigned to groups not receiving DC-CIK. There were significant differences on both PFS and OS between these two groups ($P < 0.05$, Fig. 2C and D).

Predictors associated with clinical outcomes

Cox proportional hazards models were 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 in univariate analysis. After adjusting for competing risk factors, the therapeutic modality of DC-CIK combined with S-1 plus cisplatin was confirmed to be an independent predictor of OS (HR,0.336; 95% CI, 0.261–0.752; $P = 0.001$.) TNM stage and ECOG-PS were also associated with adverse prognosis in patients with AGC (HR, 1.571; 95% CI,

Table 1. Demographics and baseline characteristics of patients

Variable	S-1 alone group	S-1 plus cisplatin group	DC-CIK combined with S-1 group	DC-CIK combined with S-1 plus cisplatin group	*P*
Case, *n*	18	15	17	13	
Age	60.1 ± 11.4	62.4 ± 14.6	61.8 ± 13.7	62.1 ± 10.6	0.415
Sex					0.966
Female	12	9	10	8	
Male	6	6	7	5	
ECOG-PS					0.754
0	12	10	9	8	
1	5	5	8	4	
2	1	0	0	1	
TNM staging					0.718
III	6	3	6	5	
IV	12	12	11	8	
Disease status					0.851
Unresectable	14	12	14	9	
Recurrent	4	3	3	4	
Previous adjuvant chemotherapy					0.385
Yes	2	4	2	4	
No	16	11	15	9	
Histopathological type					0.853
Papilary adenocarcinoma	6	7	10	8	
Tubular adenocarcinoma	7	5	4	4	
Poorly differentiated adenocarcinoma	2	1	2	0	
Mucinous adenocarcinoma	1	1	1	1	
Others	2	1	0	0	
Number of organs involved					0.819
1	6	5	8	5	
2	8	9	6	6	
>2	4	1	3	2	
Site of metastasis					0.957
Lymph node	7	8	5	6	
Liver	4	3	5	3	
Lung	2	2	1	3	
Peritoneum	1	3	2	1	
Bone	3	1	1	2	
Other	0	2	1	1	

1.286–4.023; $P = 0.007$ and HR, 1.432, 95% CI, 1.230–2.721, $P = 0.008$; Supplementary Table S1).

Phenotypic analysis of peripheral blood immune cells in patients with treatment of DC-CIK

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 ($P < 0.05$), whereas the $CD8^+/CD28^-$, $CD4^+/CD25^+$ and NKT cell subsets were significantly decreased after DC–CIK cell therapy ($P < 0.05$; Fig. 3). Similarly, during the second cycle of DC–CIK therapy, $CD3^+$, $CD8^+/CD28^+$ T-cell subsets remained at higher levels consistent with a continuous cellular immunity recovery. The $CD3^+/CD8^+$ were concurrently elevated ($P < 0.05$) whereas suppressor T cells of $CD8^+/CD28^-$ and $CD4^+/CD25^+$ remained stable at the decreased levels seen in cycle 1 (data not shown).

Genome alterations in ctDNA

We sequenced serial ctDNA in the patients who received treatment with DC–CIK. gDNA was also obtained and sequenced. About 10 Gb and 2 Gb sequencing data were generated for each ctDNA sample and gDNA sample, respectively. The average coverage of depth was 1,323-fold (706–2,094) for ctDNA samples. Somatic SNVs and Indels were identified on the basis of these sequencing data (Supplementary Table S2). To determine the associations of the alterations of ctDNA with subsequent treatment responses to DC–CIK infusions, we have analyzed molecular tumor burden in serial blood samples of these patients. Among the 30 patients who received DC–CIK infusions, there

Table 2. The disease control rate of different groups

	S-1 alone group		S-1 plus cisplatin group		DC-CIK combined with S-1 group		DC-CIK combined with S-1 plus cisplatin group		
Variables	No.	(%)	No.	(%)	No.	(%)	No.	(%)	*P*
DCR	1	5.6	5	33.3	8	47.1	10	76.9	0.001
CR	0	0	0	0	0	0	1	7.7	
PR	0	0	1	6.6	2	11.8	2	15.4	
SD	1	5.6	4	26.7	6	35.3	7	53.8	
PD	17	94.4	10	66.7	9	52.9	3	23.1	

Abbreviations: CR, complete response; DCR, disease control rate; PD, progressive disease; PR, partial response; SD, stable disease.

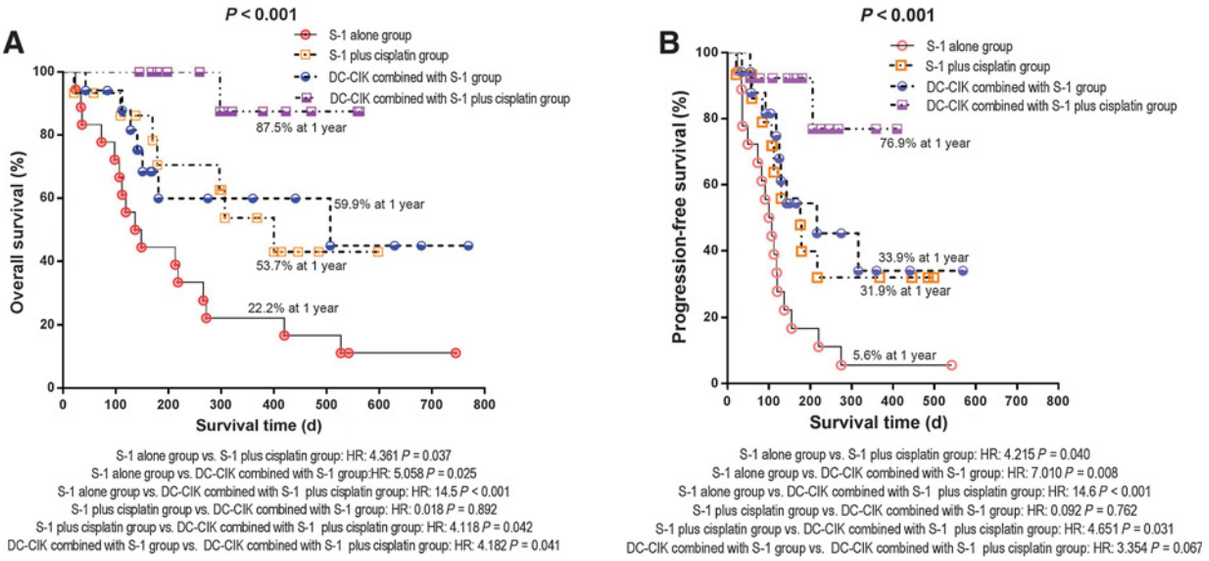

Figure 1.
A, Overall survival (OS) and **B,** Progression-free survival (PFS) for the different treatment groups.

were 85 gene mutations including *APC, ALK, NOTCH, PETN, TP53 TSC2,* and others and similar mutation profile between the two groups of patient with DC–CIK therapy (DS vs. DSC = DC–CIK combined with S-1 group vs. DC-CIK combined with S-1 plus cisplatin group; Supplementary Fig. S1). The frequencies of gene mutations were determined pre-and post-DC–CIK and we found that 19 patients (63.3%) had a decrease in number of gene mutations and frequency of ctDNA mutations (and 11 did not). Patients with a decrease in ctDNA had a relatively superior OS and PFS compared with those who did not experience a decrease in ctDNA mutations).

Variable TCR diversity after expansion is observed and associated with outcomes in patients with AGC

Cultured T-cell samples of 17 of 30 patients were qualified and feasible for performing next generation sequencing of the CDR3 of the T-cell receptor chain. The number of T-cell subclones increased after expansion *in vitro* (Fig. 4A). Importantly, the changing of TCR repertoire was significantly associated with both ctDNA mutational number and frequency in patients received treatment of DC–CIK (Fig. 4B). Moreover, as shown in Fig. 4C and D, the unique TCR subclones increased and the shared TCR subclones decreased after T-cell expansion *in vitro* in 9 patients, whereas as shown in Fig. 4E and F, the unique TCR subclones decreased and the shared TCR subclones increased after T-cell expansion *in vitro* in 8 patients. Further survival analysis showed that the unique TCR subclones increasing was related with the prognosis of patients with AGC. We showed that significantly longer OS and PFS in patients in whom the unique TCR subclones increased compared with those in whom the unique TCR subclones decreased (Fig. 4G and H; $P < 0.05$).

Treatment toxicity

We recorded all acute and delayed adverse events from the four groups (Supplementary Table S3). The majority of the toxicities are those expected from chemotherapy. The S-1 plus cisplatin group experienced more occurrences of leucopenia, neutropenia, anemia, and thrombocytopenia than the S-1 alone group, indicating that addition of cisplatin producing more toxic reactions. Among nonhematologic adverse events, there were no significant differences were shown in various groups. Moreover, there were no unanticipated significant differences in adverse events between the groups with and without treatment of DC–CIK cell immunotherapy. In particular, there were no immune mediated adverse events.

Discussion

AGC remains an incurable condition and only modest progress has been made in identifying new drugs that lengthen survival (36). S-1–based regimens are widely used as they are effective and tolerable as first-line treatment of advanced GC in both Asian and Western countries (37). Previous studies showed that S-1 combination therapy is superior to S-1 monotherapy in terms of efficacy, and patients with poor prognosis disease characteristics may benefit most from S-1 combination therapy (38). Cancer immunotherapy has shown potential efficacy in tumor growth control and patient survival (39). Recently, the combination of anti–PD-1 combined with S1 was reported to achieve a response rate of 68.4% (26/38, CR 10, PR 16, ESMO 2017 Abstracts:671P) in the first line of AGC setting. Studies have shown that DC-CIK combined with different chemotherapy regimens for treatment of GC shows better efficacy than that shown by treatment with chemotherapy alone (40). In the present study, we have combined DC–CIK cell immunotherapy with S-1 plus cisplatin and found that the 1-year PFS and OS rates were significantly higher than that in the DC–CIK combined with S-1, S-1 plus cisplatin and S-1 alone groups. Because S-1 plus cisplatin is standard first line regimen for AGC, this suggests that the DC–CIK immunotherapy can add to the clinical benefit of standard multiple-agent therapy. However, we also noted that DC/CIK combined with S-1 did not provide a statistically significant benefit compared with S1 with cisplatin ($P = 0.892$). Because not all patients are able to tolerate the toxicity of multiagent chemotherapy but that the DC–CIK was

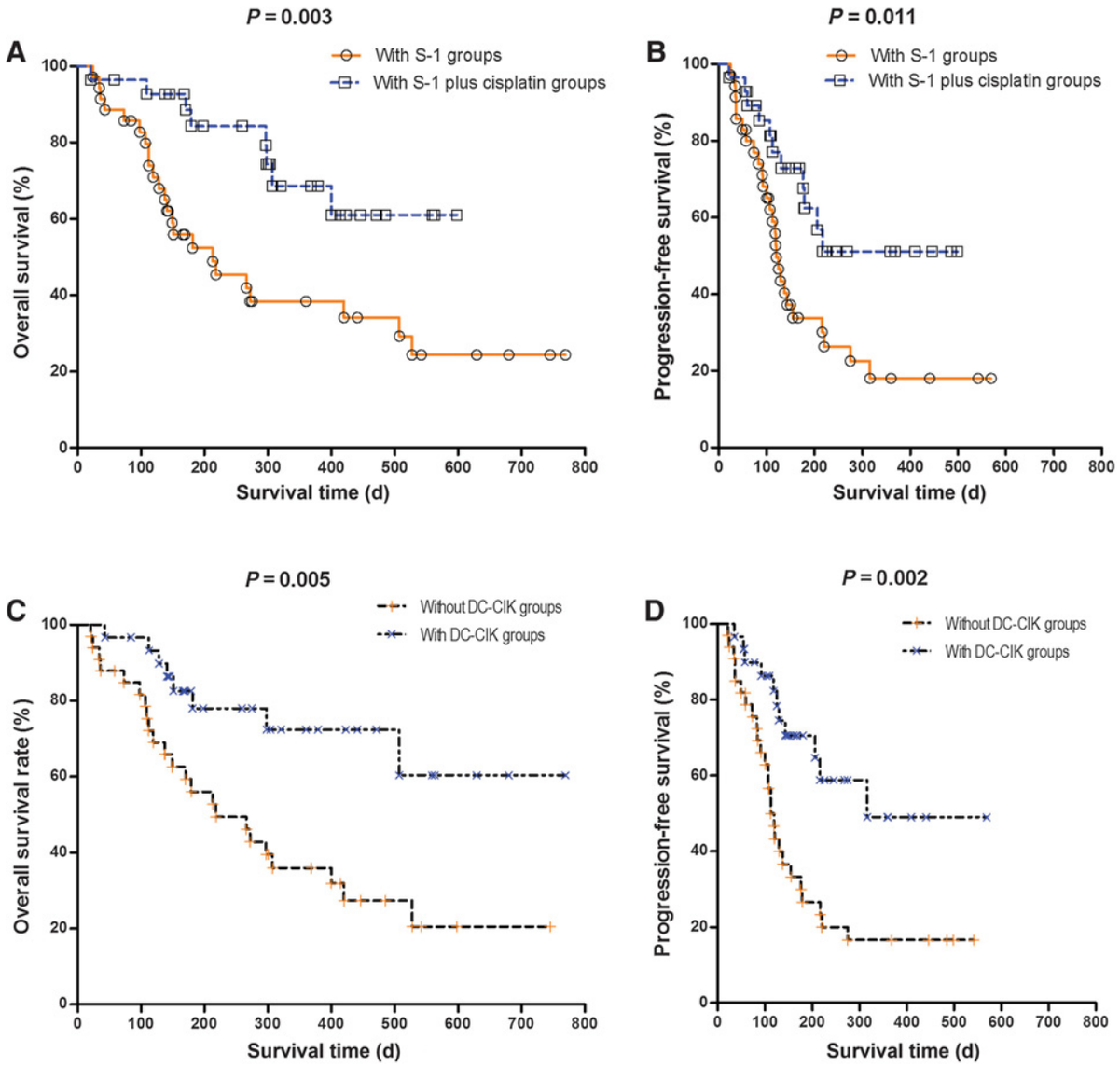

Figure 2.

A, OS and **B,** PFS for patients who received S-1 plus cisplatin and S-1 alone. **C,** OS and **D,** PFS for patients who were treated without and with DC-CIK cell therapy.

well tolerated, this suggests that DC/CIK plus S-1 could be an alternative to S1 plus cisplatin when multiple-agent chemotherapy is not tolerable.

DC–CIK immunotherapy delivers both dendritic cells 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 some concerns 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, without any serious adverse reactions (41, 42). With the development of cancer immunotherapy, there have been conceptually recognition that combined therapeutics are accepted. For the conventional chemotherapy, the interactions between chemotherapeutic regimens with immuno-therapeutic modulation have also been noticed, in which cytotoxic drugs could attack tumor cells and subsequently affect the tumor micro-environment, resulting in tumor antigen release from tumor tissue, potentially expose more antigen epitope, upregulation dendritic cell presentation. Meantime, such interactions could also interfere with tumor microenvironment to trigger the reshaped vascularization to let cytotoxic T-cell circulate into tumor cells. In the present study, 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 ($P < 0.05$), whereas the $CD8^+/CD28^-$, $CD4^+/CD25^+$ and NKT cell subsets were significantly decreased after DC–CIK cell therapy ($P < 0.05$). Therefore, the combination of S-1–based regimens with DC-CIK favorably modulates the immune milieu of the host.

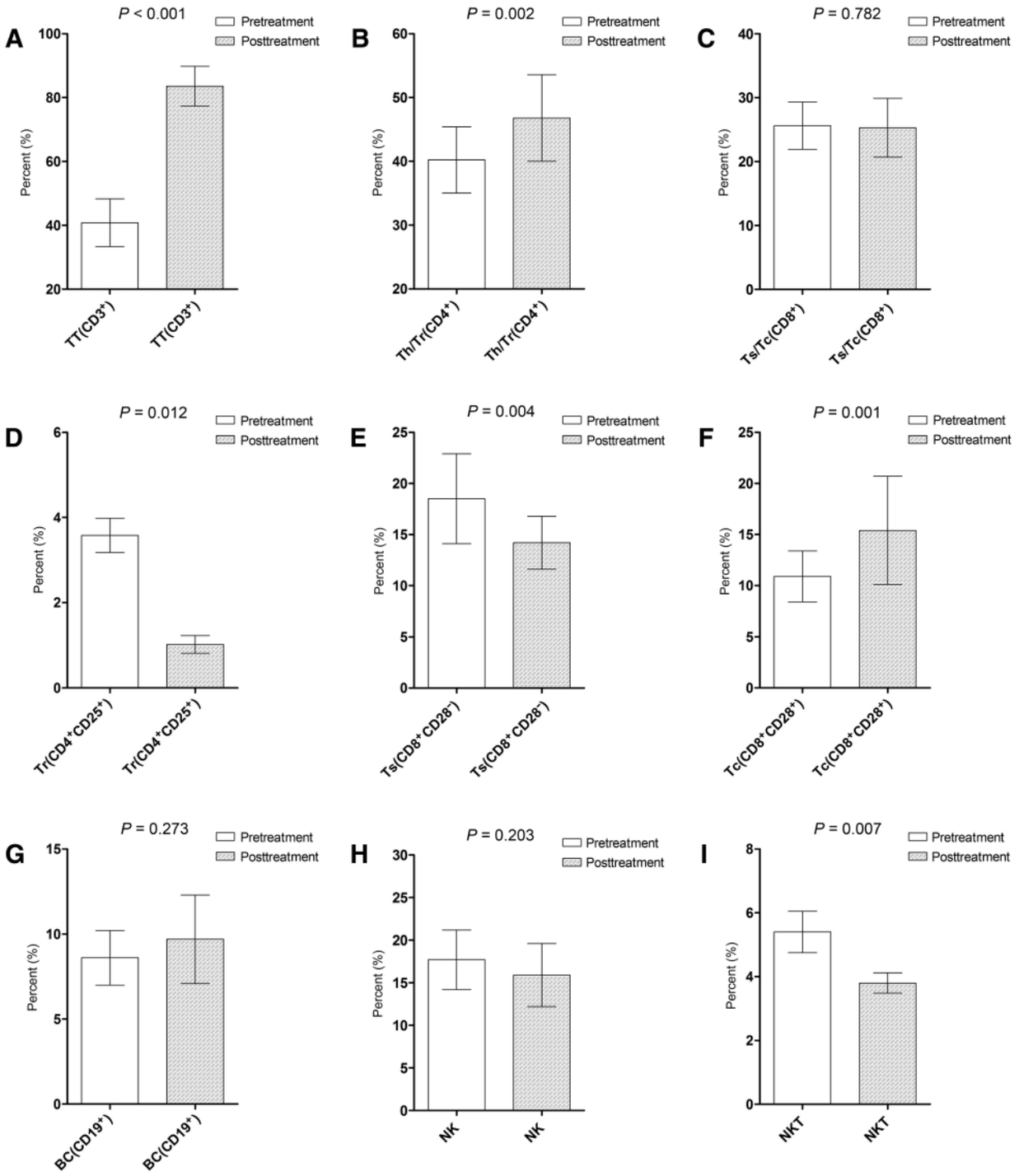

Figure 3.

Peripheral blood T-cell phenotype measurements via cytometry before and after the first cycle of DC-CIK cell therapy. $CD3^+$ (**A**), $CD3^+/CD4^+$ (**B**), and $CD8^+/CD28^+$ (**F**) T cell subsets were significantly increased after DC-CIK cell therapy ($P < 0.05$); $CD4^+/CD25^+$ (**D**), $CD8^+/CD28^-$ (**E**) and NKT (**I**) cell subsets were significantly decreased after DC-CIK cell therapy ($P < 0.05$); $CD3^+/CD8^+$ (**C**), $CD19^+$ (**G**) and NK (**H**) cell subsets were not significantly changed after DC-CIK cell therapy ($P > 0.05$).

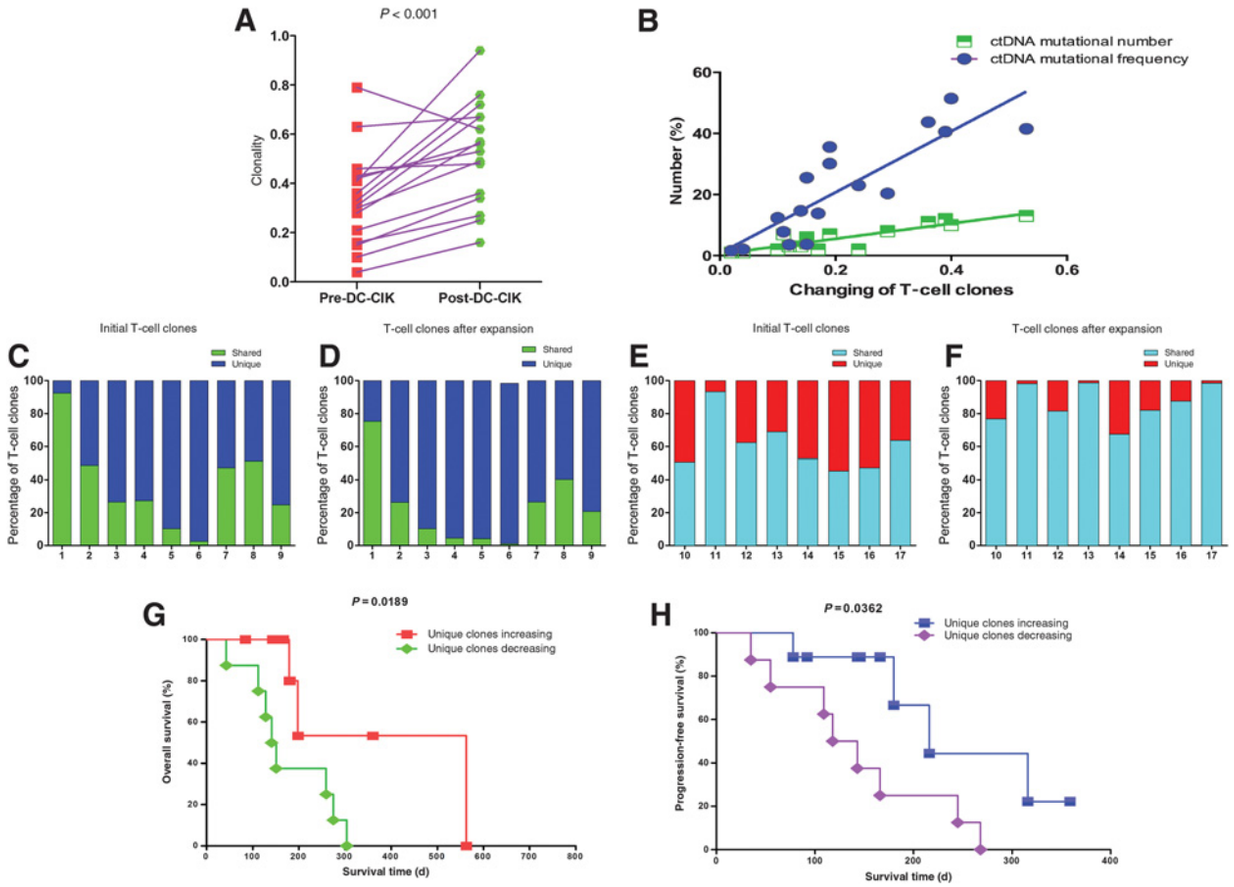

Figure 4.

A, The clonality of T cells from pre- and post-DC–CIK therapy was analyzed after *in vitro* expansion. **B,** Correlation of the change in TCR repertoire versus change in ctDNA mutational number and frequency in patients who received DC-CIK. **C** and **D,** Unique TCR clones increased and the shared TCR clones decreased after T-cell expansion *in vitro* in 9 patients ($P < 0.05$). **E** and **F,** the unique TCR clones decreased and the shared TCR clones increased after T-cell expansion *in vitro* in 8 patients ($P < 0.05$). **G** and **H,** Survival analysis of patients in whom the unique TCR clones increased compared with those in whom the unique TCR clones decreased.

Nucleic acids are released and circulate in the peripheral due to apoptosis and necrosis of cells. During tumorigenesis there is an increase in cell turnover and thus more cell necrosis and apoptosis such that tumor DNA is released into the blood stream (ctDNA). ctDNA has been exploited as a cancer biomarker. High plasma ctDNA content is associated with poor survival in patients with different malignancies and the concentration of ctDNA correlates strongly with clinical outcome (43, 44). Importantly, hypermutated ctDNA has served as a predictive biomarker for patients receiving immunotherapy (45). In this study, we sequenced ctDNA to evaluate for molecular aberrations in a panel of genes relevant for immune responses and cancer signaling pathways. The gene panel we applied is about 1.1 Mb, which include coding exons and selected introns of 1,021 genes. Among these mutated genes, IL6ST is related to Foxp3(+)CD8(+) T-cell development and function (46). IL7R is an intrinsic biomarker predicting the fate of $CD8^+$ T effectors (47). *JAK*, *PIK3*, *PTEN*, *STAT3* and *NOTCH* related signaling pathways are associated with T-cell differentiation and $CD8^+$ T-cell and immune-checkpoint inhibitor-mediated antitumor immune responses (48, 49).

As in our previous study (21), we have observed that there is clinical utility for ctDNA analyses as a multipurpose biomarker for patient response to treatment with DC–CIK cell therapy. Critically, our ctDNA analyses demonstrated that 85 mutations were detected and 19 patients (63.3%) had a decrease in the number and frequency of these mutations after treatment with DC–CIK infusions. These data provided evidence that changes in ctDNA somatic mutation allele frequency during therapy may predict response and emergence of resistance mechanisms, thus permitting selection of patients who might benefit from DC–CIK immunotherapy.

There are several limitations in this study predominantly marked by the small sample size and non-randomized design. In this study, the treatment decision was made by both physician and communications with the patients. In the S1 alone subgroup, the administration for including cisplatin was not fully according to the comorbidities and ECOG. It was based our study design on patients' enrollment because the superiority of the combination of S1 with cisplatin (from SPIRITS trial) and the adverse effects of combination with cisplatin were introduced by the physicians. Nevertheless, previous exposures of chemotherapy and the

tolerance of cisplatin hesitated the repetitive administration of such chemo regimen, which were considered by the patients. As the popularity of shared decision-making (SDM) is emerging, both the patient and physician contribute to the medical decision-making process. Health care providers explain treatments and alternatives to patients to provide the necessary resources for patients to choose the treatment option that best aligns with their unique cultural and personal beliefs. Furthermore, we are encouraged to work with the statistician and bioethicist to figure out pros and cons of such clinical design, even CAR–T-cell therapy was FDA approved on the basis of on the single arm, non-randomized study. Although the toxic profile is favorable to the DC–CIK therapy, it is difficult of performing this therapy in the standard clinical practice, as the results in this study should be validated by randomized controlled trials with large amount of patients, and more valuable prospective immuno-biomarkers should be further investigated for patients who are sensitive to the treatment of ACT.

In conclusion, the present study confirmed the favorable PFS and OS of DC–CIK combined with S-1 plus cisplatin in patients with AGC. Clinical efficacy correlated with decreases in ctDNA mutational profiles and changes in TCR repertoire.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

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Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): G. Qiao, M.A. Morse, J. Song, X. Xia, J. Ren
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Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): X. Wang, L. Zhou, X. Zhou, Y. Song, J. Ren
Study supervision: X. Wang, L. Zhou, X. Xia, J. Ren

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