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ORIGINAL ARTICLE

Autologous cytokine-induced killer cell immunotherapy in lung cancer: a phase II clinical study

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Abstract
Objective Cytokine-induced killer (CIK) cells have the ability to kill tumor in vitro and in vivo. This study was designed to evaluate the clinical efficacy of CIK cell immunotherapy following regular chemotherapy in patients with non-small cell lung cancer (NSCLC) after surgery.
Methods A paired study, with 87 stage I–IV NSCLC patients in each group, was performed. Patients received either chemotherapy (arm 2) or chemotherapy in combination with autologous CIK cell immunotherapy (arm 1). Progression-free survival (PFS) and overall survival (OS) were evaluated.
Results Of the 87 paired patients, 50 had early-stage disease (stage I–IIIA) and 37 had advanced-stage disease (stage IIIB–IV). Among early-stage patients, the distribution of 3-year PFS rate and median PFS time showed no statistical difference between the two groups ($p = 0.259$ and 0.093, respectively); however, the 3-year OS rate and median OS time in arm 1 were significantly higher than those in arm 2 (82 vs. 66 %; $p = 0.049$ and 73 vs. 53 months; $p = 0.006$, respectively). Among the advanced-stage patients, the 3-year PFS and OS rates of arm 1 were significantly higher than those of arm 2 (6 vs. 3 %; $p < 0.001$ and 31 vs. 3 %; $p < 0.001$, respectively); the median PFS and OS times in arm 1 were also significantly longer than those in arm 2 (13 vs. 6 months; $p = 0.001$ and 24 vs. 10 months; $p < 0.001$, respectively). Multivariate analyses indicated that the frequency of CIK cell immunotherapy was significantly associated with prolonged PFS (HR = 0.91; 95 % CI 0.85–0.98; $p = 0.012$) and OS (HR = 0.83; 95 % CI, 0.74–0.93; $p = 0.001$) in the arm 1.
Conclusions The data suggested that CIK cell immunotherapy could improve the efficacy of conventional chemotherapy in NSCLC patients, and increased frequency of CIK cell treatment could further enhance the beneficial effects. A multi-center randomized trial is being carried out in our hospital to further validate these findings.

Keywords Lung cancer · Cytokine-induced killer cell · Immunotherapy · Prognosis

Disclaimers: This study has not been presented in part anywhere.

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Introduction

Lung cancer was the most commonly diagnosed cancer and the leading cause of cancer death in men in 2008

globally. In women, it was the fourth most commonly diagnosed cancer and the second leading cause of cancer death. Lung cancer accounts for 13 % (1,600,000) of all cancers and 18 % (1,400,000) of the deaths in 2008 in the world [1]. Approximately, 85 % of all lung cancer cases are categorized as non-small cell lung cancer (NSCLC), and more than 50 % of NSCLC patients have advanced local invasion and/or distant metastases, which need post-operative treatments including chemotherapy, radiotherapy, and immunotherapy [2]. The current standard therapeutic regimen for patients with advanced NSCLC is platinum-based doublet chemotherapy, and an additional cytotoxic agent does not provide additional clinical benefits but only increases the toxicity [3]. Meta-analysis of several randomized trials has demonstrated a modest survival advantage of cisplatin-based regimens in patients with advanced stage of NSCLC [4]. The Eastern Cooperative Oncology Group (ECOG) conducted a large (N = 1,207) randomized study that compared four platinum-based doublet chemotherapy regimens in NSCLC patients [5]. None of the regimens was found to yield superior efficacy, and the median survival in this study was 7–9 months. Based on these previous observations, it is concluded that an efficacy plateau is reached in advanced NSCLC patients when conventional chemotherapy is used alone. New targeted agents such as sunitinib, sorafenib, vandetanib, and bevacizumab have been developed and are used as first-line therapy in many centers. The median survival of advanced-stage NSCLC treated with these agents was approximately 10–15 months [6–8]. Although these targeted therapies represent a major advance in the treatment of NSCLC, they are palliative treatments and rarely produce durable complete remissions. These limited successes indicate that further efforts are needed to improve the current therapeutic modalities and to explore novel therapies for NSCLC, to improve patient care and increase survival.

Immunotherapy has recently become the fourth important treatment modality for malignant tumors, ranked after surgery, radiotherapy, and chemotherapy [9–11]. A number of adoptive immunotherapies using various killer cells have been reported, including lymphokine-activated killer cells (LAK) [12], tumor infiltrating lymphocytes (TIL) [13], and anti-CD3 monoclonal antibody-induced killer cells [14]. However, their therapeutic efficacy is limited due to their low antitumor activities [15]. At present, cytokine-induced killer (CIK) cells have been recognized as a new type of anti-tumor effector cells, which can proliferate rapidly in vitro, with stronger anti-tumor activity and broader spectrum of targeted tumors than other reported anti-tumor effector cells [9, 16]. Moreover, CIK cells can regulate and generally enhance the immune functions in cancer patients [17]. Current data from phase I/II studies on the anti-NSCLC effects of CIK cells are highly limited, and the therapeutic benefits of CIK cells are unknown in NSCLC. The purpose of this phase II study is to evaluate the clinical efficacy of CIK cell treatment in patients with NSCLC after surgery.

Patients and methods

Patients

We performed a paired study to evaluate the clinical outcomes of CIK cell immunotherapy in naive and primary patients with stage I–IV NSCLC. This study was approved by the State Food and Drug Administration of China (2006L01023) and by the ethics committee of Cancer Hospital of Tianjin Medical University, according to the guidelines of the Declaration of Helsinki. Informed consent was obtained from all patients. The criteria for patient selection included age between 18 and 80 years, expected survival duration of >3 months, a Karnofsky performance status (KPS) score >40 %, and free of cardiac arrhythmias, congestive heart failure, and severe coronary artery disease. Pregnant and lactating women were excluded. The patients were continuously recruited from January 1, 2003 to March 1, 2008 and were re-diagnosed according to the NCCN Clinical Practice Guidelines [18]. Patients received either chemotherapy (arm 2) or chemotherapy in combination with autologous CIK cell adoptive immunotherapy (arm 1) after operation and before disease progression. Eighty-seven patients were enrolled in each group (Table 1). Patients in the two groups were matched for clinical stage, histology, sex, age, neutrophils, platelets (PLT), hemoglobin (HGB), lactate dehydrogenase (LDH), β2-microglobulin (β2-MG), KPS and smoking index at diagnosis, the time of diagnosis, operation and treatment, and subsequent therapies. The follow-up started from January 1, 2003 and ended on April 1, 2011.

Treatments

All patients in the two groups received chemotherapy with TP regimen (paclitaxel, 135 mg/m^2, day 1; cisplatin, 80 mg/m^2, day 1), GP regimen (gemcitabine, 1,000 mg/m^2, days 1 and 8; cisplatin, 80 mg/m^2, day 1), or NP regimen (navelbine, 25 mg/m^2, days 1 and 8; cisplatin, 80 mg/m^2, day 1). Patients in arm 1 received each cycle of chemotherapy on day1 or day1, 8, and following CIK infusion on day15, 16 at an interval of 1 month. For each treatment, patients were treated with intravenous infusions of $(13.07 \pm 1.37) \times 10^9$ CIK cells at days 15 and 16 of each

Table 1 Distributions of demographic and clinical characteristics of patients in the two groups

Demographic and clinical features	Stage I-IIIA		*p* value	Stage IIIB–IV		*p* value
	Arm 1	Arm 2		Arm 1	Arm 2	
No. of patients	50	50	–	37	37	–
Median frequency of CIK treatment (range)	8 (3–34)	–	–	5 (3–15)	–	–
Sex			0.517			0.572
Male	33	36		30	28	
Female	17	14		7	9	
Age, years			0.841			0.636
≤60	25	24		21	23	
>60	25	26		16	14	
Smoking index			0.414			0.338
<400	18	22		12	16	
≥400	32	28		25	21	
KPS			0.894			0.761
≥80	48	47		19	15	
<80	2	3		18	22	
Hemoglobin			0.183			0.307
≥LLN	39	44		32	30	
<LLN	11	6		5	7	
Neutrophils			0.774			0.478
≤ULN	42	44		32	30	
>ULN	8	6		5	7	
Platelets			0.875			0.528
≤ULN	42	43		31	29	
>ULN	8	7		6	8	
LDH			0.727			1.000
≤ULN	46	45		30	30	
>ULN	4	5		7	7	
*β*2-MG			0.357			1.000
≤ULN	42	46		33	34	
>ULN	8	4		4	3	
Histology			0.611			0.862
Adenocarcinoma	30	32		23	23	
Squamous carcinoma	17	16		11	10	
Large cell	3	2		3	4	
Subsequent therapy			0.663			0.640
Radiotherapy	10	11		11	8	
Chemotherapy	30	30		4	5	
Immunotherapy	15	17		9	7	
Target therapy	3	5		4	5	

KPS Karnofsky performance status, *LLN* lower limit of normal, *ULN* upper limit of normal, *LDH* lactate dehydrogenase; *β2-MG β*2 microglobulin, – indicates not applicable

cycle. The therapeutic effect was evaluated after 2 cycles. Maintenance treatment continued unless progression of disease occurred. Patients of arm 2 received further 4 cycles of chemotherapy and patients of arm 1 received not only 4 cycles chemotherapy but also continuous CIK treatment. At least 3 treatments were completed in patients of arm 1.

Clinical assessment

Patients were assessed by oncology specialists for a complete blood count, computed tomography of chest, abdomen, and pelvis, and technetium bone scan. Response was determined based on the National Cancer Institute's Response Evaluation Criteria in Solid Tumors (RECIST)

[19]. Responding and stable patients were followed up once every other month until disease progression or as clinically indicated.

CIK cell preparation

CIK cells were prepared as described in our previous studies [20–22]. Briefly, PBMC were collected from lung cancer patients after surgery using a Cobe Spectra Apheresis System (CaridianBCT, Lakewood, CO, USA), and cultured in X-VIVO 20 serum-free medium (Cambrex, East Rutherford, NJ, USA) containing 50 ng/mL anti-CD3 antibody (Ab) to stimulate CIK cell growth, 100 U/mL recombinant human interleukin (IL)-1α (e-Bioscience, San Diego, CA, USA), and 1,000 U/mL recombinant human interferon (IFN)-γ (Peprotech, Rocky Hill, NJ, USA), at 37 °C with 5 % CO_2 for 24 h. Then, 300 U/mL recombinant human IL-2 (Peprotech) was added to the media. IL-2- and IFN-γ-containing medium was added to the culture system every 5 days. On day 14, CIK cells were harvested and analyzed for phenotype and cytotoxicity. Safety testing was performed during the course of cell culture. All products were free of bacterial and fungal contamination, negative for mycoplasma, and contained <5 Eu endotoxin. The viability of CIK cells was usually 90–95 %.

Detecting the phenotype of CIK cells

The phenotype of CIK cells was detected as described in our previous studies [20–22]. Briefly, 5×10^5 CIK cells were resuspended in 20 μL 2 % newborn calf serum and 1 % sodium azide in phosphate-buffered saline (PBS) and incubated with 10 μL Ab against CD3-FITC/CD56-RPE (Dako, Glostrup, Denmark), CD3-FITC, CD4-RPE, and CD8-RPE (BD Bioscience, San Jose, CA, USA) for 30 min at 4 °C. After incubation, cells were washed twice with PBS and resuspended in 1.0 mL staining buffer (BD Pharmingen, Franklin Lake, NJ, USA). The cell population was analyzed using flow cytometry (BD Aria, San Jose, CA, USA).

Detecting cytotoxicity of CIK cells

The cytotoxicity of CIK cells was detected as described in our previous studies [20–22]. Briefly, the target cells used for this assay included the lung cancer cell line A549 and CALU-6, breast cancer cell line MCF-7, colon cancer cell line HCT-8, and lymphoma cell line Raji. Target cells (1×10^5 cells/mL) were incubated for 4 h in triplicate sets with effector cells (CIK cells) at a ratio of effector to target cells of 40:1. At the end of incubation, 50 μL culture supernatant was transferred to a new, flat 96-well plate and incubated with 50 μL LDH substrate mixture (for detection of LDH released upon cell lysis) at room temperature for 30 min in dark. Then, 50 μL stop solution was added to each well. Absorbance was measured at 490 nm using a 96-well plate reader. Specific cytotoxicity was calculated as: % specific cytotoxicity = [(experimental counts—effector spontaneous counts—target spontaneous counts)/(target maximal counts—target spontaneous counts)] × 100.

Statistical methods

The definitions of overall survival (OS) and progression-free survival (PFS) were referred to the RECIST [19]. OS was calculated from the time of surgery until death, and patients alive were censored at the time of last contact. PFS was calculated from the date of surgery until first progression, and patients alive in stable state were censored at the time of last contact. The χ^2 test and Fisher's exact test were used for binary variable comparisons. The Mann–Whitney U test was used for median comparisons. Distributions of survival time and rate were determined by the Kaplan–Meier method; median survival time and 3-year survival rate along with 95 % confidence intervals (CIs) were reported. Associations between survival and potential prognostic factors were assessed using the log-rank test in univariable analyses. The Cox proportional hazards model was undertaken in multivariable analyses using the Forward-LR method with a significance level of 0.15 for entering and removing variables. In univariate evaluations of the prognostic impact of continuous variable (the frequency of CIK cell treatment), the optimal cutpoint was determined using the ROC Curve method. A p value less than 0.05 using two-sided tests indicates statistical significance. All calculations were performed using the SPSS 16.0 software.

Results

Patient characteristics

Of the 87 paired patients in the two groups, 23 had stage I disease, 11 had stage II, 16 had stage IIIA, 7 had stage IIIB, and 30 had stage IV disease. The distributions of patient characteristics are shown in Table 1. The proportion of patients' clinical stage, histology, sex, age, neutrophils, PLT, HGB, LDH, β2-MG and KPS at diagnosis, time of diagnosis, operation and treatment, and subsequent therapies were comparable between the two groups (Table 1).

Analysis of CIK cells' phenotype

Phenotypic analysis of CIK cells in 87 patients from arm1 before and after 14–16 days of culture demonstrated that the percentages of $CD3^+$, $CD3^+CD4^+$, $CD3^+CD8^+$, $CD3^+CD56^+$ was significantly increased from

49.51 ± 3.56 %, 28.72 ± 4.92 %, 19.56 ± 6.79 %, and 3.78 ± 1.08 % to 82.11 ± 10.48 %, 44.22 ± 12.15 %, 36.97 ± 15.78 %, and 18.62 ± 5.57 %, respectively, with p values < 0.01. Furthermore, the population of $CD3^-/CD16^+CD56^+$ cells decreased from 13.15 ± 2.58 % to 6.59 ± 2.13 %, with p values < 0.05.

Cytotoxicity assays of CIK cells in vitro

The cytotoxicity of cultured CIK cells against human erythroleukemic cell line K562, breast cancer cell line MCF-7, colon cancer cell line HCT-8, and lymphoma cell line Raji was 44.19 ± 5.11 %, 25.92 ± 3.83 %, 27.65 ± 2.79 %, and 35.14 ± 3.28 %, respectively. Additionally, the cytotoxicity of the CIK cells from patients in arm 1 against the lung cancer cell line A549 and CALU-6 was 32.56 ± 4.11 % and 24.95 ± 2.91 %.

Prognosis of all patients in the two groups

The 3-year PFS and OS of all patients were 36 % (95 % CI 33–39 %) and 50 % (95 % CI 46–54 %), respectively. The median PFS and OS of all patients were 17 months (95 % CI 12–22 months) and 34 months (95 % CI 22–46 months), respectively. The 3-year PFS and OS in arm 1 were significantly higher than those in arm 2 ($p = 0.050$ and 0.001, respectively). Furthermore, the median PFS and OS in arm 1 were significantly longer than those in arm 2 ($p = 0.028$ and 0.001, respectively) (Table 2; Fig. 1).

Prognosis of early-stage patients in the two groups

Stratified analysis revealed that the distributions of 3-year PFS rate and median PFS time of patients with early-stage disease (stage I–IIIA) in the two groups have no statistical difference ($p = 0.259$ and 0.093, respectively). However, the 3-year OS rate and median OS time of early-stage patients in arm 1 were significantly higher than those in arm 2 (82 vs. 66 %; $p = 0.049$ and 73 vs. 53 months; $p = 0.006$, respectively) (Table 2).

Prognosis of advanced-stage patients in the two groups

The CIK cell immunotherapy in combination with chemotherapy improved the prognosis of patients with advanced-stage disease (stage IIIA–V) when compared with chemotherapy alone. The 3-year PFS and OS of advanced-stage patients in arm 1 were significantly higher than those in arm 2 ($p < 0.001$ and < 0.001, respectively). The median PFS and OS of patients with advanced-stage disease in arm 1 were also significantly longer than those in arm 2 ($p = 0.001$ and < 0.001, respectively) (Table 2; Fig. 2).

Frequency of CIK cell treatment and prognosis of patients

The median frequency of CIK cell immunotherapy was 6 times (range of 3–34 times) in arm 1. The frequency of CIK cell treatment significantly improved the prognosis of patients when analyzed as a continuous variable in the multivariate analysis after adjustment for clinical stage, pathological type, sex, age, neutrophils, PLT, HGB, LDH, β2-MG, KPS, smoking index, and other therapies (Table 3, 4). All 13 potential predictive covariates with their univariate analyses are presented in Table 3. The optimal cutpoint of the frequency was 7 times. The median PFS of 43 patients who received CIK cell treatments for ≥7 times

Table 2 Distributions of estimated 3-year PFS and OS rate, median PFS and OS time in the two groups

Clinical stage	Arm 1	Arm 2	p value
All, No.	87	87	–
3-year PFS, % (95 % CI)	39 (36–42)	32 (30–34)	0.050
3-year OS, % (95 % CI)	61 (55–67)	39 (36–42)	0.001
Median PFS, months (95 % CI)	24 (14–34)	12 (8–16)	0.028
Median OS, months (95 % CI)	48 (29–67)	18 (11–25)	0.001
Early-stage, No.	50	50	–
3-year PFS, % (95 % CI)	64 (58–70)	54 (48–60)	0.259
3-year OS, % (95 % CI)	82 (74–90)	66 (59–73)	0.049
Median PFS, months (95 % CI)	NR	41 (6–76)	0.093
Median OS, months (95 % CI)	NR	58 (49–67)	0.006
Advanced-stage, No.	37	37	–
3-year PFS, % (95 % CI)	6 (5–7)	3.0 (2.7–3.3)	<0.001
3-year OS, % (95 % CI)	31 (28–34)	3.0 (2.8–3.2)	<0.001
Median PFS, months (95 % CI)	13 (11–15)	6 (4–8)	0.001
Median OS, months (95 % CI)	24 (18–30)	10 (7–13)	<0.001

PFS prognosis-free survival, *OS* overall survival, *NR* not reached

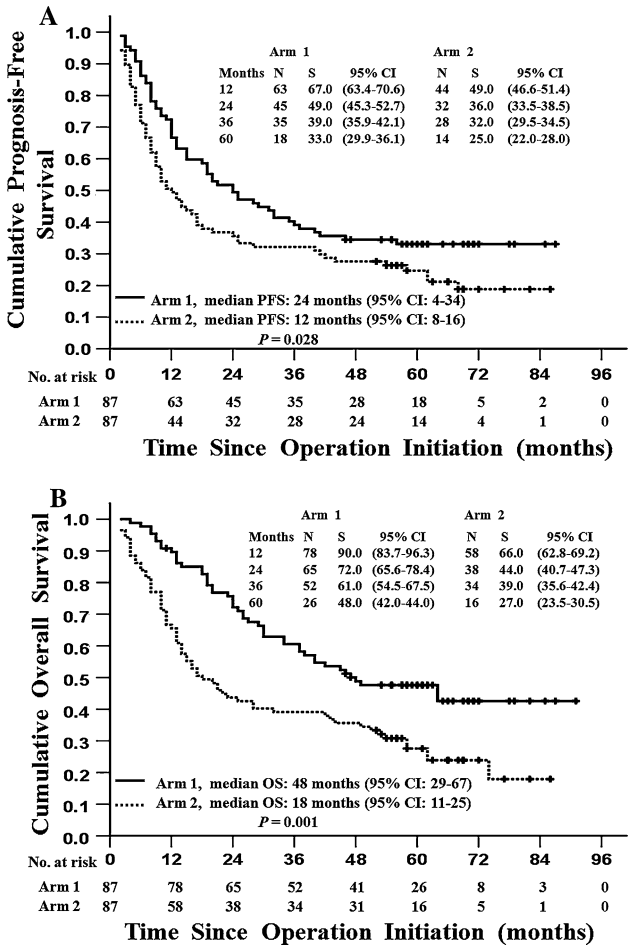

Fig. 1 Kaplan–Meier curves for progression-free survival (PFS) and overall survival (OS) in patients of arm 1 and arm 2. An event is defined as disease progression or death without progression in PFS and as death from any cause in OS. *Panels* are as follows: **a** PFS, **b** OS. In each graph, arm 1 is indicated by a *solid line* and arm 2 is indicated by a *dashed line*. *N* number at risk; *S* survival percent, with 95 % confidence interval in parentheses

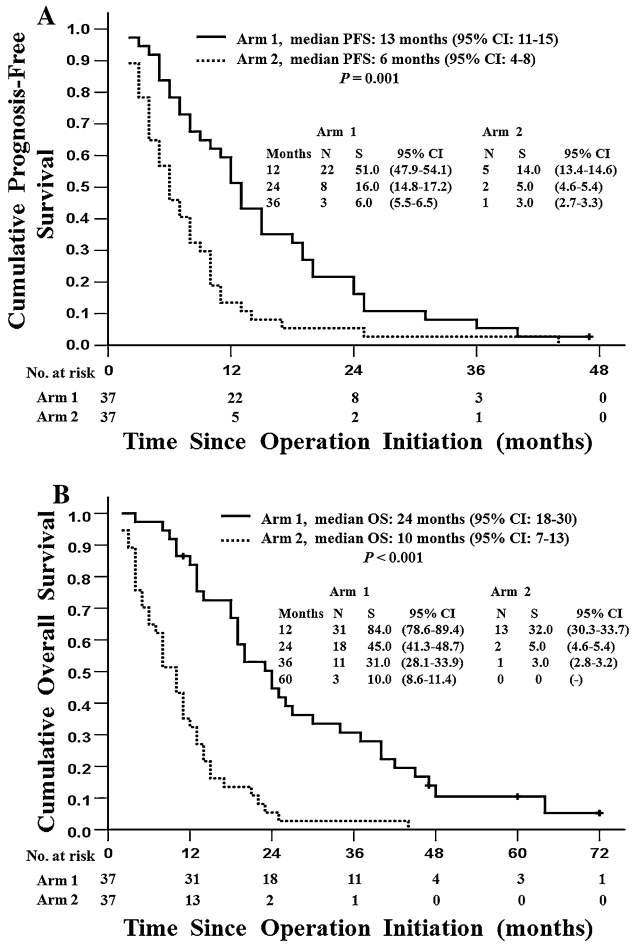

Fig. 2 Kaplan–Meier curves for progression-free survival (PFS) and overall survival (OS) in advanced-stage patients of arm 1 and arm 2. An event is defined as disease progression or death without progression in PFS and as death from any cause in OS. *Panels* are as follows: **a** PFS, **b** OS. In *each graph*, arm 1 is indicated by a *solid line* and arm 2 is indicated by a *dashed line*. *N* number at risk; *S* survival percent, with 95 % confidence interval in parentheses. – indicates not applicable

(41 months; 95 % CI was not applicable) was significantly longer than that of 44 patients who received <7 times of the treatments (13 months; 95 % CI 5–21 months) (HR = 0.30; 95 % CI 0.14–0.62; $p = 0.001$). The median OS of patients who received CIK cell treatments for ≥7 times (not reached) was significantly longer than that of patients who received the treatments for <7 times (26 months; 95 % CI, 21–31 months) (HR = 0.17; 95 % CI 0.07–0.41; $p < 0.001$) (Fig. 3).

Discussion

Two conventional adoptive cell immunotherapies are through the use of LAK cells and TILs [15, 23]. LAK cell treatment in combination with IL-2 has been extensively studied and was demonstrated to be heterogeneous and capable of killing both allogeneic and autologous tumors [24]. The activity of LAK cells was mainly mediated by NK cells and also indirectly by major histocompatibility complex (MHC) unrestricted T cells [24]. TILs represent part of the host immune response to human malignancy and contain an enriched population of cells with both cytotoxic and helper functions that are reactive to the autologous tumor [25]. The majority of TILs expanded by IL-2 are composed of both $CD3^+CD4^+$ and $CD3^+CD8^+$ T cells [25]. In addition, TILs have been demonstrated to contain antigen-specific as well as non-specific cytotoxic lymphocytes [26]. However, their therapeutic efficacy is limited due to their low anti-tumor activities [15]. CIK cells are a novel population of immune effector cells and are activated T cells with natural killer (NK) properties that can be expanded in vitro in the presence of rhIL-2, starting from

Table 3 Univariate analysis of 87 patients' demographic and clinical characteristics and survival in the arm 1

Parameters	Median OS (months)	Log-rank *p*	Median PFS (months)	Log-rank *p*
Sex		0.415		0.946
Male	45		21	
Female	64		28	
Age		0.366		0.573
<60 years	64		25	
≥60 years	38		19	
Clinical stage		<0.001		<0.001
Stage I + IIIa	NR		NR	
Stage IIIb + IV	24		13	
Histology		0.875		0.843
Adenocarcinoma	48		25	
Squamous carcinoma	46		24	
Large cell	47		22	
Cycle count of CIK treatment		<.001		<0.001
>7 cycles (cutoff)	NR		41	
≤7 cycles (cutoff)	26		13	
Smoking index		0.563		0.412
<400	63		28	
≥400	47		19	
KPS		<0.001		<0.001
≥80	49		25	
<80	18		12	
Hemoglobin		0.070		0.087
≥LLN	42		25	
<LLN	13		10	
Neutrophils		0.076		0.215
≤ULN	45		25	
>ULN	12		10	
Platelets		0.143		0.300
≤ULN	47		25	
>ULN	29		16	
LDH		0.017		<0.035
≤ULN	64		25	
>ULN	34		15	
β-MG		0.254		0.302
≤ULN	49		28	
>ULN	33		20	
Subsequent therapy		0.483		0.437
Yes	64		26	
No	45		22	

NR not reached, *KPS* Karnofsky performance status, *LLN* lower limit of normal, *ULN* upper limit of normal, *LDH* lactate dehydrogenase, *β2-MG* β2 microglobulin, *PFS* progression-free survival, *OS* overall survival

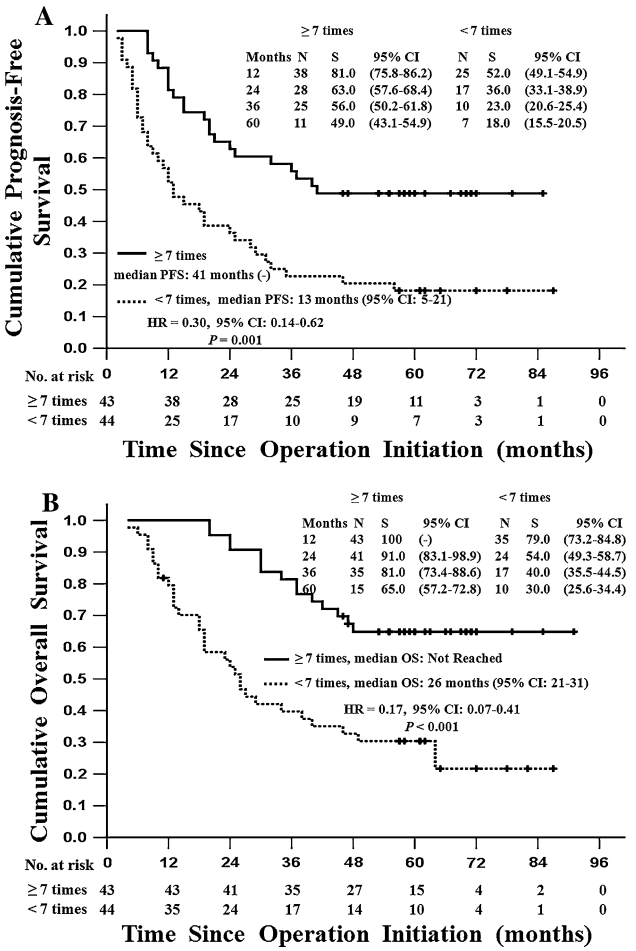

Fig. 3 Prognostic impact of the frequency of CIK cell treatment on patients in arm 1. An event is defined as disease progression or death without progression in progression-free survival (PFS) and as death from any cause in overall survival (OS). *Panels* are as follows: **a** PFS, **b** OS. In each graph, patients who received CIK cell treatments for ≥7 times are indicated by a *solid line* and patients who received CIK cell treatments for <7 times are indicated by a *dashed line*. *N* number at risk; *S* survival percent, with 95 % confidence interval in parentheses. HR = hazard ratio, with 95 % confidence interval. – indicates not applicable

peripheral blood mononuclear cells stimulated by IFN-γ and anti-CD3 antibody [16]. CIK cells express CD3 and CD56 as well as the NKG2D antigen, and show MHC-unrestricted cytotoxicity toward neoplastic but not normal targets [17, 27, 28]. CIK cells express several chemokine receptors and are shown to migrate to the tumor site after intravenous administration in in vivo models [29–31]. At the tumor site, CIK cells can exert their cytotoxic activity and control tumor growth. CIK cells can proliferate rapidly in vitro, with stronger anti-tumor activity, broader target tumor spectrum, and lower adverse effects than other reported anti-tumor effector cells [9, 16]. Moreover, CIK cells can regulate and enhance the immune function in cancer patients [17]. Their ease of production in vitro and anti-tumor potential have made them suitable candidates

Table 4 Multivariable analysis of 87 patients' demographic and clinical characteristics and survival in the arm 1

Parameters	OS			PFS		
	Hazard ratio	95% CI	*p* value	Hazard ratio	95% CI	*p* value
CIK treatments >7 cycles	0.17	0.07–0.41	<0.001	0.30	0.14–0.62	0.001
Clinical stage IIIb + IV	8.83	4.38–17.81	<0.001	5.02	2.47–9.19	<0.001
KPS < 80	4.18	1.41–12.50	0.010	2.53	1.35–5.47	0.029
LDH > ULN	2.33	1.05–5.65	0.054	1.26	0.95–3.47	0.068

KPS Karnofsky performance status, *ULN* upper limit of normal, *LDH* lactate dehydrogenase, *PFS* progression-free survival, *OS* overall survival

for cell therapy regimens in solid and hematopoietic tumor treatments. Indeed, both autologous and allogeneic CIK cells have been employed in phase I/II clinical trials for the treatment of various tumor types. In these trials, they have shown limited in vivo toxicity and evidence of anti-tumor activity [20–22, 32–35]. However, current knowledge on the anti-NSCLC effects of CIK cells in phases I/II studies is highly limited [21, 34, 35]. Our previous study showed that dendritic cell-activated CIK cells enhance the anti-tumor effect of chemotherapy in NSCLC patients after surgery [21]. To our knowledge, the present report is the largest prognostic study in NSCLC treated with CIK cell immunotherapy. By stratifying analysis in this paired study, we have shown that CIK cell immunotherapy could improve the effect of chemotherapy in NSCLC patients.

In the multivariate analysis, the frequency of CIK cell treatment is significantly associated with prognosis in the CIK group, when analyzed as a continuous variable. Previous studies have demonstrated that the minimal time for immunotherapy to display an effect in cancer patients is about 8 months [36, 37]. In our study, the optimal cutpoint of the frequency of CIK therapy was determined to be 7 times. The prognosis of patients who received CIK cell treatments for $\geq$7 times was significantly better than that of patients who received <7 times of the treatments. The median time of the 7th treatment was 9.5 months (range of 8–12 months). These data indicate that the time CIK cell immunotherapy starts to display an effect in NSCLC is approximately 10 months, and the maintenance treatments are required after the initial effect is observed for maximal benefits. However, the preferred length for maintenance treatments is still unclear and needs further investigations. Multi-center randomized trial is needed to confirm the minimum number of treatments, the time for an effect to display, and the time for maintenance treatments in NSCLC treated with CIK cells.

In conclusion, we have revealed the relationship between the CIK cell immunotherapy and the prognosis of NSCLC. Our study has indicated that CIK cell treatment could improve the prognosis of NSCLC, and increased frequency of CIK cell immunotherapy could result in additional benefits. A multi-center randomized trial in our hospital is being carried out to further validate these findings.

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Conflict of interest The authors declare that they have no conflict of interest.

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