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Cord blood-derived cytokine-induced killer cells biotherapy combined with second-line chemotherapy in the treatment of advanced solid malignancies

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ABSTRACT

This study investigated the efficacy of cord blood-derived cytokine-induced killer (CB-CIK) biotherapy combined with second-line chemotherapy in treating advanced solid malignancies after first-line chemotherapy failure. Forty patients with advanced solid malignancies after first-line chemotherapy failure were divided into two groups: CB-CIK cells transfusion plus second-line chemotherapy (CB-CIK + Chemotherapy) group and second-line chemotherapy alone (Chemotherapy) group. The ORR and DCR were 30% and 80% in CB-CIK + Chemotherapy group compared with 15% and 70% in Chemotherapy group ($P = 0.451$ for ORR and $P = 0.716$ for DCR) respectively. The time to progression and the median survival time were 3.45 months (95% CI 2.30–4.60 months) and 11.17 months (95% CI 9.05–13.28 months) in CB-CIK + Chemotherapy group compared with 2.03 months (95% CI 1.23–2.82 months) and 7.52 months (95% CI 5.97–9.06 months) in Chemotherapy group respectively. Compared with patients in Chemotherapy group, the patients in CB-CIK + Chemotherapy group had significantly longer PFS ($P = 0.031$) and overall survival ($P = 0.048$). In vitro studies further revealed that CB-CIK cells could overcome drug resistance in cisplatin-resistant lung adenocarcinoma cell line A549/CDDP through down-regulating ABCG-2 and P-gp and induce cytotoxicity through the high level expression of CD3, CD56, FasL, and CD69. This could explain why CB-CIK could have synergistic effects with second-line chemotherapy shown in this clinical study. We concluded CB-CIK cells combined with second-line chemotherapy can significantly improve PFS and median survival compared with second-line chemotherapy alone in patients with advanced solid malignancies after first-line chemotherapy failure.

1. Introduction

For advanced solid malignancies, the efficiency of second-line chemotherapy is often low. A main cause of the failure of chemotherapy is the resistance of tumor cells against anticancer compounds, and a major form of such a resistance is called multidrug resistance (MDR), caused by the increased expression of ABC (ATP binding cassette) multidrug transporter proteins, which are active in an ATP-dependent manner extruding a large variety of drugs or drug conjugates from the cells. ABC proteins comprise a large superfamily of transmembrane proteins [1–3]. By far the best known major drug transporters, i.e. ABCB1 (P-glycoprotein, p-gp or MDR1), ABCG2 (BCRP), ABCC1 (MRP1), and ABCC2 (MRP2, cMOAT) have been characterized in detail with respect to their structure and function [4–6]. P-glycoprotein (p-gp) encoded by the mdr-1 gene is a 170 kDa plasma membrane protein that functions as an ATP-driven drug export pump. Cytotoxic drugs of natural origin with different chemical structures and mechanisms of action, such as vinca alkaloids, anthracyclines, epipodophyllotoxins and taxanes, can be extruded by p-gp through the cell membranes of resistant cells and cross-resistance occurs [7]. ATP-binding cassette protein G2 (ABCG2) is a 655-amino-acid polypeptide transporter that forms a homodimer and has been reported as a tetramer in plasma membranes. ABCG2 can extrude the antineoplastic drugs such as anthracyclines and camptothecins, which plays an important role in chemotherapy drug resistance [8].

New anticancer approaches to overcome chemotherapy drug resistance and increase survival for advanced solid malignancies who failed to first-line chemotherapy are needed. Immunotherapy stimulating the immune system and enhancing the patient's own anti-tumor ability is an alternative and promising way to treat cancer and overcome chemotherapy drug resistance [9,10]. In the 1980s, the lymphocytes activated by IL-2, were used for treating malignancies at advanced stage [11–13]. Since then, several kinds of immunological effector cells such as lymphokine-activated killer (LAK) cells, tumor infiltrating lymphocytes (TIL), anti-CD3 induced activated killer cells (CD3AK) and cytokine-induced-killer (CIK) cells have been used as immunotherapy, and a protocol by which CIK cells were considerably induced in vitro was developed [12,14–20]. CIK cells are MHC-unrestricted cytotoxic lymphocytes generated by incubation of peripheral blood lymphocytes with anti-CD3 monoclonal antibody,

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IL-2, IL-1 and IFN-γ. CIK cells represent the lymphocytes with increased anti-tumor cytotoxic activity in vitro and in vivo [18], and high proliferation ability as compared to LAK cells [21,22]. The increase of lysis activity to tumor cells is mainly due to the high proliferation potential of CD3 and CD56 double positive cells in CIK cells [19,21,23,24]. CIK cells are therefore suitable for the immunotherapy against advanced solid malignancies. Cord blood (CB) is progressively becoming an extensively used treatment for patients with both malignant and nonmalignant disorders. The CIK cells obtained from CB have an overlapping phenotype with the traditional CIK cells obtained from adult peripheral blood. Both CD3 + CD56− and CD3 + CD56+ cells contribute to their cytotoxicity. The CD3 + CD56+ cells, derived from CD3 + CD56− cells, could expand by up to 1000-fold and gave the greatest cytotoxicity against various tumor cell targets, as compared to CD3 + CD56− cells [25]. The activation of T lymphocytes, both in vivo and in vitro, inducing expression of CD69 is involved in lymphocyte proliferation and functions as a signal-transmitting receptor in lymphocytes, such as CIK cells [26]. Fas ligand (FasL or CD178), a type-II transmembrane protein, binding with its receptor induces target cell apoptosis. Fas ligand/receptor interactions play an important role in the regulation of the immune system, the progression of cancer and the apoptosis of carcinoma cells inducted by T lymphocytes [27]. HLA-DR is a major histocompatibility complex, MHC class II, cell surface receptor encoded by the human leukocyte antigen complex. The primary function of HLA-DR is to present peptide antigens, potentially foreign in origin, to the immune system for the purpose of eliciting or suppressing T-(helper)-cell responses that eventually lead to the production of antibodies against the same peptide antigen [28]. CD25 is the alpha chain of the IL-2 receptor. The IL-2/IL-2R interaction then stimulates the growth, differentiation and survival of antigen-selected cytotoxic T cells via the activation of the expression of specific genes [29]. In addition, they express NKG2D and perforin, the two proposed molecules which have been demonstrated to play an important role in CIK-mediated cytotoxicity so far [30,31]. Studies by Nishimura et al. showed that adoptive transfer of allogeneic CIK cells caused minimal graft-versus-host disease (GVHD) because CIK cells infiltrated GVHD target tissues much less and transiently [32]. CIK cells produce large amounts of IFN-γ, which has protective effects against GVHD [33]. Moreover, the cord blood-derived cytokine-induced killer (CB-CIK) cells have been shown to be able to kill variety of human solid and hematological malignancies both in vitro and in vivo [34,35]. Clinical studies with CB-CIK cells to treat malignancies showed promising efficacy with negligible side effects such as transient fever and Myalgia which mostly disappeared even without needs of anti-inflammatory drug administration [36]. All these suggest that CB-CIK cells have great potentials to be clinically used in treating human malignancies as a safe and effective approach especially for advanced cancer patients who cannot tolerate chemotherapy or radiotherapy. Recently, Deng et al. reported that CB-CIK cells alone have been used in treating advanced human lung cancer with minimal side effects and some efficiency [36].

Currently, CIK cells transfusion has evolved to become a promising way for overcoming chemotherapy resistance [9,10]. Liu et al. reported that CIK cells possessed a higher antitumor cytotoxic activity against a drug-resistant lung adenocarcinoma cell line in vitro and they found CIK cells plused with docetaxel demonstrated a prominent augmentation of antitumor activity against multidrug resistance lung adenocarcinoma cell lines both in vitro and in vivo [10]. Since most of the clinical studies so far showed that the efficiencies of CIK cells especially autologous ones to treat human malignancies were low [37,38] and studies have showed that CB-CIK cells are more potent in cytotoxic activity against various tumor cells than autologous ones with similar phenotype both in vitro and in vivo, we hypothesized CB-CIK cells could act synergistically with chemotherapeutic agents on malignant cells after first-line chemotherapy failure and CB-CIK cells might help second-line chemotherapeutic agents to overcome cancer cells' drug resistance, and thus improve PFS and median survival of patients with advanced solid malignancies. To test this hypothesis, we performed a randomized block design study to evaluate the potential effect of CB-CIK combined with second-line chemotherapy on survival, response evaluation and tolerability in advanced solid malignancies after first-line chemotherapy failure. Also, we performed in vitro studies to reveal the potential role of CB-CIK in this combination therapy.

2. Materials and methods

2.1. CB-CIK induction

The mononuclear cells were prepared from cord blood with Ficoll-Hypaque centrifugation method, and then induced with the recombinant cytokines IFN-γ at 1000 U/ml (Boehringer Mannheim, Germany), IL-2 at 300 U/ml (Boehringer Mannheim, Germany), IL-1 at 100 U/ml (Boehringer Mannheim, Germany) and anti-CD3 antibody at 50 ng/ml (Boehringer Mannheim, Germany) for 15 days. CB-CIK cell growth was observed under the microscope and CB-CIK cells phenotypes were determined by flow cytometry [31]. Unless the CB-CIK population contains more than 35% of CD3 + CD56+ cells, it can't be permitted for clinical treatment.

2.2. Study population and treatment groups

Adults suffering from metastatic solid tumors were enrolled in the protocol and a written informed consent was provided in accordance with the Declaration of Helsinki. Adult patients with histologically documented solid malignancies who failed to first-line chemotherapy were potentially eligible for this study. Patients were excluded if, based on the clinical judgment of the treating physician, they had a life expectancy of less than 1 month, had an indication for liver failure, renal failure, thromboembolism, or autoimmune disease. Eligible patients were asked for written informed consent. Information collected at baseline included WHO performance status, and type, histology, stage, and duration of cancer. Protocol design, data collection, and analysis were solely the responsibility of the authors.

There are 40 patients with advanced solid malignancies after first-line chemotherapy failure were enrolled and divided into two groups in this study. One group received second-line chemotherapy plus CB-CIK treatment (CB-CIK + Chemotherapy group), the other group received standard second-line chemotherapy alone (Chemotherapy group). It is a randomized block design study, meanwhile, the balance of malignancies kinds and other characters between the two groups was considered.

2.3. Treatment regimens

Standard second-line chemotherapy regimens were used individually according to the NCCN guidelines. Two cycles of chemotherapy were performed for every patient. CIK cells were cultivated using cord blood cells. About 9×10^9 CB-CIK cells in 100 ml NS, followed by 5 mg Dexamethasone, were transfused into patient every time. One week after chemotherapy administration, patients who received second-line chemotherapy combined with CB-CIK cells therapy received CB-CIK cells for a total of 6 times within 12 days.

2.4. Follow-up

The discharged patients were told to contact us if new symptoms were found, and come back within 2 months for review. For the period after the planed treatment finished, the effects of treatment were evaluated every 2 or 3 months until death. A standardized

questionnaire was used to obtain information about survival and treatment efficiency. All patients were followed-up until death or until the end of the study, with a minimum of 2 months of follow-up.

2.5. Outcome measures

The primary end point was death as a result of any cause. The primary safety outcomes were divided into two categories. CB-CIK treatment-related side effects including GVHD-related and others were observed in this study. Chemotherapy-related side effects including myelosuppression, gastrointestinal reaction, fever, fatigue, and rash were compared between the two groups.

2.6. Cancer cell lines

Human leukemic cell lines K562 and multidrug resistant human lung adenocarcinoma cell subline-A549/CDDP, which are induced by Cisplatin (DDP) showed resistance to Cisplatin and also showed cross-resistance to Carboplatin (CBP) and Etoposied (Vp-16) [39]. Cisplatin-resistant lung adenocarcinoma cell lines A549/CDDP were obtained from the tumor cell bank of Chinese Academy of Medical Sciences (Chaoyang District, Beijing, China). Cell lines were grown in RPMI medium supplemented with 10% human AB serum. Then 2 μmol/L cisplatin (Qilu Pharmaceutical Co, Ltd, Shandong, China) was added to the medium of the A549/CDDP cell line. A549/CDDP cells were cultured in complete DMEM medium without cisplatin for 3 days before being used in experiments.

2.7. Phenotype analysis

After ex vivo expansion for 5, 14 and 18 days, CIK cells were harvested from culture. For cell phenotype identification, CIK cells were incubated for 30 min with mAb against CD3, CD56, HLA-DR, CD25, CD69, FasL (Biolegend, San Diego, CA), coupled to FITC or PE. CIK cells were analyzed on a FACSCalibur flow cytometer (BD Bioscience, CA) using the CellQuest software (BD Bioscience, CA)/WinMDI statistical software (Scripps, La Jolla, CA).

For p-gp, ABCG2 identification, A549/CDDP cells were incubated for 30 min with mAb against p-gp, ABCG2 (Biolegend, San Diego, CA), coupled to FITC or PE. A549/CDDP cells were analyzed on the FACSCalibur flow cytometer (BD Bioscience, CA).

2.8. Cytotoxicity activity assay of CB-CIK against cancer cells

One hundred microliters of target cells were seeded in a microplate at a 1×10^5/mL density and 0.1 mL of effect cells at a 1×10^6/mL density were mixed into each well (to reduce the influence of effect cell number, we deliberately made the E:T = 10:1). After 24 h of incubation, 10 μL of MTT was added. The following treatments were the same as in drug-resistant test. Cytotoxicity rate (CR) was calculated by the following formula: $CR=\{1-(A_{570\ experiment}-A_{570\ E\ control})/A_{570T\ control}\}\times100\%$.

2.9. Expression changes of p-gp and ABCG2 in A549/CDDP cells induced by CB-CIK

Effectors (CB-CIK, 2×10^6 cells/ml) and targets (A549/CDDP or K562, 2×10^5 cells/ml) were incubated in 96-well plates at an effector-to-target ratio of 10:1. After 8 h, cells were stained with Annexin V-FITC (Annexin V-FITC apoptosis detection kit, BioVision research product, Linda Vista Avenue, Mountain View, CA) and analyzed on a FACSCalibur flow cytometer (BD Bioscience, CA).

After 6 h of incubation with CB-CIK, A549/CDDP cells were incubated for 30 min with mAb against p-gp, ABCG2 (Biolegend, San Diego, CA), coupled to FITC or PE. A549/CDDP cells were analyzed on the FACSCalibur flow cytometer (BD Bioscience, CA) again.

2.10. Statistical analysis

All primary analyses were performed on an intention-to-treat principle. RECIST (response evaluation criteria in solid tumor) was applied to evaluate the treatment efficacy. There are four grades, CR (complete response), PR (partial response), SD (stable disease) and PD (progressive disease), in RECIST system. The RECIST analysis was calculated according to the ordered one-way data of Ridit analysis. PFS (progression-free survival) analysis was calculated according to the T-test and Levene-test. The primary analysis of survival was based on the time from the beginning of treatment to death. Patients alive at the end of follow-up were censored. Survival estimates were calculated according to the Kaplan-Meier method and COX analysis. The effect of the two kinds of treatment regimens was calculated using a two-sided log-rank test. Ninety-five percent confidence intervals were calculated when appropriate. Analysis of cytotoxicity test was performed by using Student's T test, the data were expressed as mean ± SD and $P<0.05$ was considered statistically significant.

3. Results

3.1. Population

Between May 2007 and May 2009, a total of 40 patients with advanced solid malignancies after first-line chemotherapy failure were enrolled in this study. Of these, 20 patients received second-line chemotherapy plus CB-CIK treatment (CB-CIK + Chemotherapy group), 20 patients received standard second-line chemotherapy alone (Chemotherapy group). Most of the baseline characteristics of the patients were well balanced between the two groups (Tables 1 and 2). In this study, there were 8 lung cancer patients in each group, accounting for 40% of the total patients.

3.2. Efficacy

In the intention-to-treat population, the median survival time was 11.17 months for CB-CIK + Chemotherapy group (95% CI 9.05–13.28 months) versus 7.52 months for Chemotherapy group (95% CI

Table 1
Baseline characteristics of the patients.

Characteristic	CB-CIK + Chemo (n = 20)		Chemotherapy (n = 20)	
	No. of patients	%	No. of patients	%
Age, years				
Median	60		62	
Range	35–71		38–77	
Male sex				
Weight, kg				
Median	65		64	
Range	41–105		38–94	
Interval from cancer diagnosis to inclusion, months				
Median	15		16	
Interquartile range	6–43		10–38	
Interval from metastasis diagnosis to inclusion, months				
Median	6		5	
Interquartile range	2–18		2–14	
WHO status[a]				
≤1	14	70	13	65
2	5	25	7	35
3	1	5	0	0
Life expectancy				
<6 months	11	55	10	50
≥6 months	9	45	10	50

[a] Higher scores on the WHO scale indicate poorer performance.

Table 2
Baseline cancer characteristics of the patients.

Characteristic	CB-CIK + Chemo (n = 20)		Chemotherapy (n = 20)	
	No. of patients	%	No. of patients	%
Histologic type of cancer			9	45
Adeno	10	50	4	20
Squamous	3	15	1	5
Non-small cell	1	5	2	10
Transitional cell	2	10	1	5
Small cell	1	5	2	10
Other	2	10		
Tumor stage			17	85
Metastatic disease	18	90	3	15
Locally advanced disease	2	10		
Type of cancer				
Lung	8	40	7	35
Gastric or esophageal	3	15	4	20
Colorectal	2	10	2	10
Breast	1	5	2	10
Gallbladder	1	5	0	0
Liver	1	5	1	5
Cervical	1	5	1	5
Ovarian	1	5	1	5
Melanoma	2	10	1	5
Sarcoma	0	0	1	5

5.97–9.06 months) (P = 0.048) and the time to progression was 3.45 months (95% CI 2.302–4.598 months) in CB-CIK + Chemotherapy group and 2.025 months (95% CI 1.232–2.818 months) in Chemotherapy group (Table 3). At 6 months the survival was 82% in CB-CIK + Chemotherapy group versus 53% in Chemotherapy group. For 12 months these estimates were 43% versus 22% (Fig. 1). Compared with patients in Chemotherapy group, the patients in CB-CIK + Chemotherapy group had significantly longer PFS (P = 0.031) and overall survival (P = 0.048) (Fig. 2).

Ranked data Ridit analysis for RECIST (response evaluation criteria in solid tumor) in the two groups (F = 1.28, P = 0.264) showed the ORR was 30% and 15% in CB-CIK + Chemotherapy group and Chemotherapy group respectively (P = 0.451). The DCR was 80% vs. 70% in CB-CIK + Chemotherapy group and Chemotherapy group respectively (P = 0.716) (Table 3). Response evaluation in CB-CIK + Chemotherapy group tends to be better than Chemotherapy group. However, the advantages did not reach statistical significance.

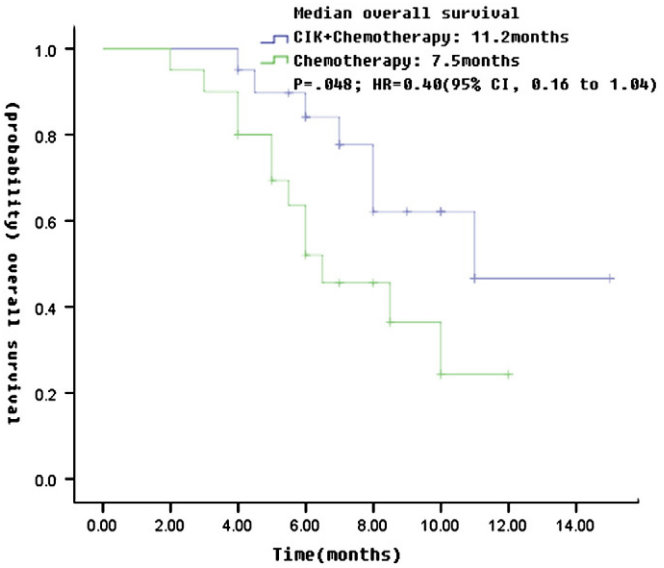

Fig. 1. Kaplan and Meier estimates of the probability of survival in cancer patients who failed to first-line chemotherapy, according to whether patients were randomly assigned to CB-CIK + Chemotherapy group or Chemotherapy group, show that the survival of CB-CIK + Chemotherapy group patients was significantly longer than those in Chemotherapy group. The P value (P = 0.048) was derived from a log-rank test comparing both treatment groups. The hazard ratio of mortality was 0.40 (95% CI, 0.16 to 1.04) in favor of the CB-CIK + Chemotherapy group.

3.3. Tolerability

Adverse events associated with CB-CIK and chemotherapy were summarized in Table 4. Fever (1 patient 5%), flu-like symptoms (1 patient, 5%), fatigue (1 patient, 5%), myalgia (1 patient 5%) were observed in 20 patients who received CB-CIK treatment. In total, GVHD-related side effects (1 patient, 5%, acute GVHD) and other side effects (3 patients, 15%). No seriously CB-CIK treatment-related adverse events were observed. Grade 3 to 5 adverse events associated with chemotherapy were observed in 9 patients (45%) in CB-CIK + Chemotherapy group and in 12 patients (60%) in Chemotherapy group. No adverse event resulting in death was observed in both groups (Table 4).

Table 3
Efficacy results.

Efficacy endpoint	CB-CIK + Chemotherapy (n = 20)			Chemotherapy (n = 20)					
	No.	%	95% CI	No.	%	95% CI	P	Hazard ratio	95% CI
Overall survival, months									
Median		11.17			7.52		.048	.40	.16 to 1.04
Range		4–15			2–12				
PFS, months									
Median		3.45			2.025		.031	.54	.28 to 1.01
Range		0–10			0–7				
Best overall response[a]							.264[b]		
CR	0	0		0	0				
PR	6	30		3	15				
SD	10	55		11	55				
PD	4	15		6	30				
Overall response rate(CR + PR)	6	30		3	15		.451[a]		
Disease control rate(CR + PR + SD at first tumor assessment)	16	80		14	70		.716[a]		

Abbreviations: CR, complete response; PR, partial response; SD, stable disease; PD, progressive disease.
n = 20 for CB-CIK + Chemotherapy; n = 20 for Chemotherapy.
[a] X^2 test.
[b] Ridit analysis.

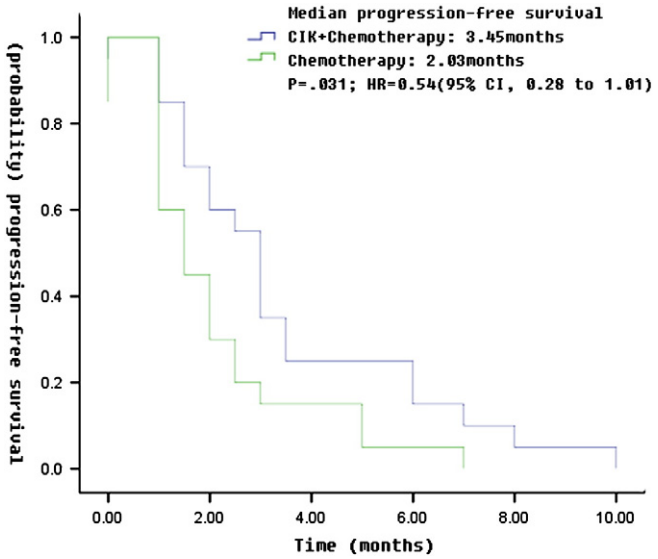

Fig. 2. Kaplan and Meier estimates of the probability of PFS (progression-free survival) in cancer patients who failed first-line chemotherapy, according to whether patients were randomly assigned to CB-CIK + Chemotherapy group or Chemotherapy group, showing that PFS of CB-CIK + Chemotherapy group patients was significantly longer than the Chemotherapy group. The P value (P = 0.031) was derived from a log-rank test comparing both treatment groups. The hazard ratio of mortality was 0.54 (95% CI, 0.28 to 1.01) in favor of the CB-CIK + Chemotherapy group.

3.4. Dynamic analysis of CB-CIK phenotypes

The dynamic changes of CB-CIK cell phenotypes in different time point of culture are shown in Tables/Figs. After in vitro expansion for 5, 14 and 18 days, CD3, CD56, HLA-DR, CD25, CD69, Fasl were analyzed on a FACSCalibur flow cytometer. The final CB-CIK population contained a mean 73.11% of CD3+/CD56+ cells, 99.47% of CD69+, 88.35% of CD178 (Fasl)+, 52.95% of HLA-DR+ and a mean 42.31% of CD25+ cells (Fig. 3).

Table 4
Most common all-grade adverse events in patients treated with chemotherapy or chemotherapy plus CB-CIK.

	CB-CIK + Chemotherapy		Chemotherapy	
	Any grade, %	Grade >2, %	Any grade, %	Grade >2, %
Adverse events of CB-CIK treatment				
GVHD-related side effects	5			
Acute GVHD-related side effects	5			
Rash	0			
Nausea and diarrhea	0			
Hypohepatia	0			
Fever	5			
Chronic GVHD-related side effects	0			
Other side effects	15			
Flu-like symptoms	5			
Myalgia	5			
Chills	0			
Fatigue	5			
Adverse events of chemotherapy				
Anemia	25	5	30	10
Thrombocytopenia	25	5	25	5
Neutropenia	15	5	25	5
Diarrhea	10	0	10	0
Nausea	35	10	55	20
Rash	0	0	10	0
Vomiting	25	10	30	10
Pyrexia	35	0	30	5
Fatigue	20	5	55	10
Anorexia	35	5	60	15
Constipation	30	10	25	10

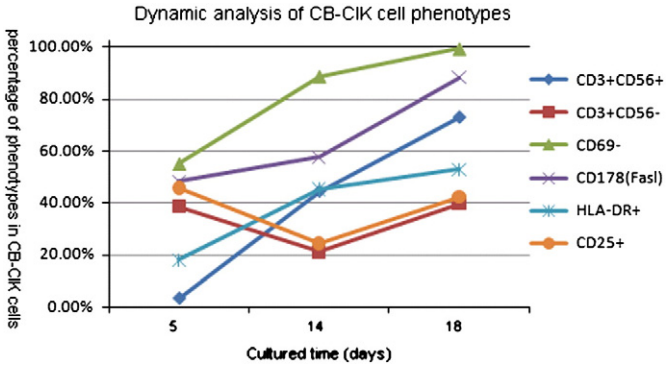

Fig. 3. The dynamics of CB-CIK cell phenotypes in different time points of culture. After in vitro expansion for 5, 14 and 18 days, CD3, CD56, HLA-DR, CD25, CD69, and Fasl were analyzed on a FACSCalibur flow cytometer. The CD3+/CD56+, CD69+ and CD178 (Fasl)+ subsets increased remarkably, in addition, the HLA-DR+ subsets increased slightly. The final CB-CIK population contained a mean 73.11% of CD3+/CD56+ cells, 99.47% of CD69+, 88.35% of CD178(Fasl)+, 52.95% of HLA-DR+ and a mean 42.31% of CD25+ cells.

3.5. Cytotoxicity of CB-CIK against multidrug resistant cancer cell line A549/CDDP and leukemic cancer cell line K562

Here we found that CB-CIK cells had similar cytotoxicity both to K562 and to A549/CDDP (Fig. 4), we chose E: T = 10:1 to avoid bias produced by effect cell number in the test. The experiment wells contained 10^4 target cells mixed with 10^5 effect cells. The cytotoxicity of CB-CIK to K562 was 46.61 ± 2.32%, while to A549/CDDP it was 45.86 ± 2.01% (P > 0.05).

3.6. Expression changes of p-gp and ABCG2 in A549/CDDP induced by CB-CIK

MDR cell line A549/CDDP cells express the p-gp, ABCG2 at high levels. After being incubated with CB-CIK, the p-pg expression of

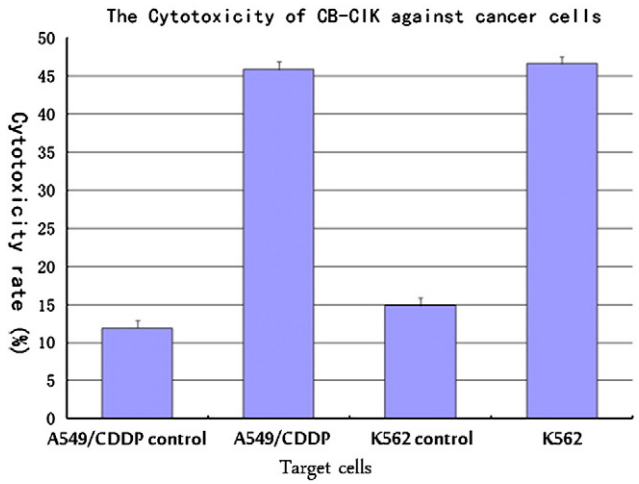

Fig. 4. Cytotoxicity of CB-CIK cells against multidrug resistant cancer cell line A549/CDDP and leukemic cancer cell line K562 were tested by MTT assay. The control samples (A549/CDDP control and K562 control) were target cells (A549/CDDP or K562) without CB-CIK cells. The cytotoxicity of CB-CIK to K562 was 46.61 ± 2.32%, while to A549/CDDP it was 45.86 ± 2.01% (P > 0.05). It means that CB-CIK cells had similar cytotoxicity both to K562 and to A549/CDDP.

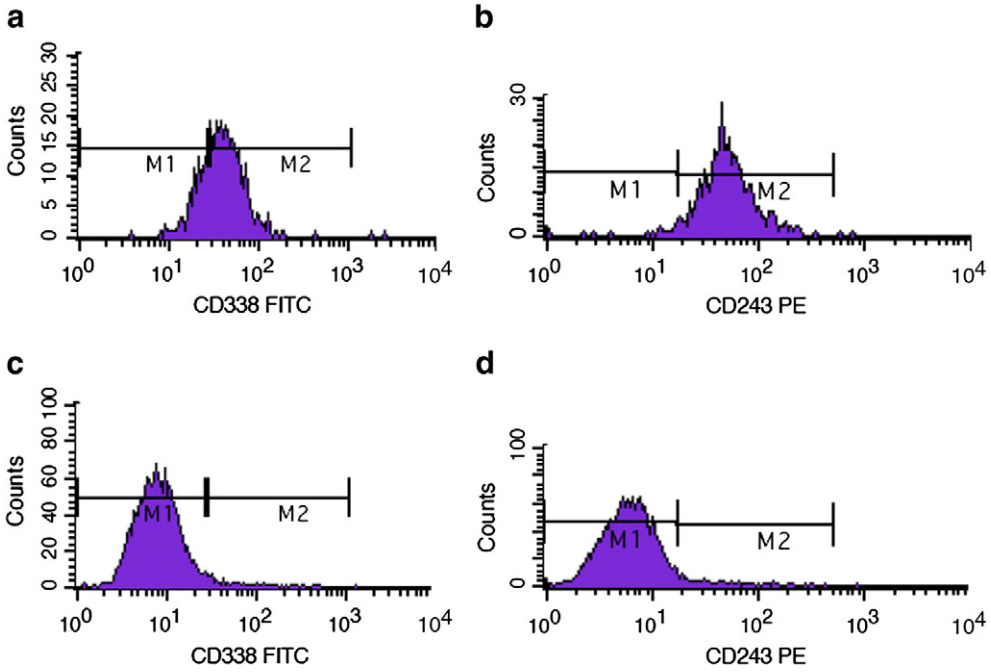

Fig. 5. Expression changes of p-gp and ABCG2 in A549/CDDP cells induced by CB-CIK for 8 h. a. Expression of ABCG2 (CD338) in A549/CDDP cells was analyzed on FACS. b. Expression of p-gp (CD243) in A549/CDDP cells without CB-CIK was analyzed on FACS. c. Expression of ABCG2 (CD338) in A549/CDDP cells induced by CB-CIK was analyzed on FACS. d. Expression of p-gp (CD243) in A549/CDDP cells induced by CB-CIK was analyzed on FACS. MDR cell line A549/CDDP cells without CB-CIK express the p-gp, ABCG2 at high levels. After being incubated with CB-CIK, both p-pg and ABCG2 expression in A549/CDDP were significantly down-regulated.

A549/CDDP decreased from a mean of 61.33% to 34.77%, and the ABCG2 expression decreased from a mean of 68.99% to 35.06%, $p<0.05$. Therefore, the expressions of p-gp and ABCG2 in A549/CDDP were significantly down-regulated after 8 h of incubation with CB-CIK, compared with their initial expressions (Fig. 5).

4. Discussions

CIK cells are a novel population of immune effect cells with high proliferation rate and potent antitumor activity against a variety of tumor cells [40]. After 18 days of incubation, our CB-CIK cells expressed high level of CD3, CD56, CD69, and FasL, which implied that mature CB-CIK cells had strong proliferation and cytotoxicity against tumor cells. The $CD3^{+}CD56^{+}$ cells gave the greatest cytotoxicity against various tumor cell targets [25]. CD69 appears to be the earliest inducible cell surface glycoprotein acquired during lymphoid activation, and is involved in lymphocyte proliferation and signal transmitting [26]. FasL, which is affected by Fas/FasL pathway, plays an important role in apoptosis of carcinoma cells inducted by CIK [27]. Autologous CIK cells have been used to treat various human malignancies including hematologic and solid ones with some efficiency. Schmidt-Wolf et al. described the first clinical trial using CIK cells for the treatment of ten patients with progressive metastatic disease resistant to chemotherapy [41]. They demonstrated the feasibility and the low toxicity of such an approach and one patient with follicular lymphoma developed a CR [37,38]. Meanwhile, some studies have showed that CB-CIK cells were more potent in cytotoxic activity against various tumor cells than autologous ones with similar phenotype both in vitro and in vivo [34,35]. However, there were few reports about the clinical application of CB-CIK cells on the treatment of advanced cancer patients. The present study evaluated the possible advantages provided by approaches that combined modalities, i.e. second-line chemotherapy plus CB-CIK immunotherapy in treating advanced cancer patients after first-line chemotherapy failure.

Our results indicate that CB-CIK cells combined with second-line chemotherapy can significantly improve PFS and median survival with minimal side effects. First-line chemotherapy failure for advanced solid malignancies often predicts short survival, and chemotherapy drug resistance as well as poor performance status. Our results of CB-CIK cells can improve PFS and median survival demonstrate the great potentials of CB-CIK cells in treating chemotherapy-resistant advanced solid malignancies. The response evaluation data in this study suggested that CB-CIK cells had a clear trend toward improved ORR when combined with second-line chemotherapy compared with second-line chemotherapy alone, even though the trend of ORR did not reach statistical significance. Considering the small population of patients in current study, further investigations are needed to confirm this trend shown in this study in larger scale of population.

The side effects of CB-CIK treatment are well tolerated by all the patients in this study. Commonly seen side effects were low level of fever, Myalgia, flu-like symptoms, and fatigue which were easily handled with a dosage of anti-inflammatory drug when the symptoms become severe. No other significant side effects were found in this study. Furthermore, CB-CIK seemed to improve the toxicities of chemotherapy as grade 3 to 5 adverse events associated with chemotherapy were lower in CB-CIK + Chemotherapy group than those in Chemotherapy group (45% versus 60%). Since all the patients in our study were in advanced stages, CB-CIK cells might be quite safe in clinical applications.

Chemotherapy and CB-CIK could have synergistic effects, and thus improve PFS and survival shown in this study. On one hand, CB-CIK could overcome drug resistance and induce cytotoxicity of cancer cells. This study showed that CB-CIK cells could overcome multidrug resistance in MDR lung adenocarcinoma cell line A549/CDDP through downregulating ABCG-2 and P-gp. Our MTT assay showed that CB-CIK cells had similar cytotoxicity both to normal cancer cell line K562 and to MDR cancer cell line A549/CDDP. Therefore, CB-CIK cells could destroy chemo-resistant cancer cells and act synergistically with chemotherapeutic agents on cancer cells. Other studies also suggested

CIK/CB-CIK cells possess strong cytotoxicity to multidrug-resistant cell line [10,42]. On the other hand, increasing evidence has been accumulated indicating that chemotherapy also can improve anti-tumor immune response and made tumor cells more susceptible to the immunotherapy. Chemotherapy can eliminate cells with immunosuppressive activity such as Myeloid-derived suppressor cells (MDSC), and regulatory T cells [43]. MDSCs play an important role in tumor escape by suppressing T cell responses [44,45]. Regulatory T cells are important negative regulators of immune responses in cancer [46,47]. The decrease in the number of MDSC and Regulatory T could dramatically improve anti-tumor immune response. Recently, Gabrilovich et al. found that chemotherapy made tumor cells more susceptible to the cytotoxic effect of CTLs through a dramatic perforin-independent increase in permeability to GrzB released by the CTLs [48,49]. Our results that CB-CIK combined with chemotherapy can improve the survival and PFS of advanced cancer patients also suggest that CB-CIK could act synergistically with chemotherapy. To our knowledge, this is the first clinical report that CB-CIK cells when combined with second-line chemotherapeutic agents could improve median survival and PFS of advanced cancer patients who failed to first-line chemotherapy.

In conclusion, these preliminary data showed that CB-derived CIK cells combined with second-line chemotherapy can significantly improve PFS and median survival with minimal side effects. CB-CIK could overcome cancer cells' multidrug resistance through down-regulating ABCG-2 and P-gp and have cytotoxic activity against multidrug-resistant cancer by high level expression of CD3, CD56, FasL, and CD69. CB-CIK cells have the great potentials to be clinically used in chemotherapy-resistant advanced solid malignancies. Further studies in larger scale are clearly needed to confirm the definite efficiency and safety of CB-CIK cells shown in this study.

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