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Efficacy and Safety of Using Autologous Dendritic Cells Combined with Cytokine-Induced Killer Cells as an Adjuvant Therapy on Advanced Malignant Melanoma

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Abstract

Adaptive immunotherapy with Dendritic Cells (DCs) combined with Cytokine Induced Killer (CIK) cells has been used as an adjuvant therapy to prolong the survival of patients who suffered with multiple solid tumors, but its benefits on Malignant Melanoma (MM) remain unclear. Our study focuses on the evaluation of the efficacy and safety of using DCs-CIK cells immunotherapy as an adjuvant therapy for MM patients undergoing conventional therapy. Altogether, 22 MM patients were enrolled in this retrospective study. Among these patients, 12 patients only received surgery, chemotherapy or radiotherapy (control group), while 10 patients received DCs-CIK immunotherapy after surgery, chemotherapy or radiotherapy (DCs-CIK group). Kaplan-Meier and Cox regression analyses were conducted to explore differences in Overall Survival (OS) and Progression-Free Survival (PFS) of patients in the two groups. The 1- and 2-year OS rates of patients in DCs-CIK group were 90.0% and 45.0%, respectively, significantly higher than 50.0% and 0 in control group (P=0.015). The 1 and 2 year PFS rates of patients in DCs-CIK group and control group were 88.9% and 25.0%, respectively, significantly higher than 25.0% and 0 in control group (P=0.030). Further analysis based on ulcer showed that there were no significant differences in OS and PFS of patients with ulcer in the two groups (POS=0.072, PPFS=0.072). For patients without ulcer, patients in DCs-CIK group exhibited better OS and PFS than patients in control group (POS=0.039, PPFS=0.023). No serious adverse events were observed in this study. After conventional therapy, DCs-CIK immunotherapy may be an effective and safe adjuvant treatment for melanoma patients, especially for those without ulcer.

Keywords: Malignant melanoma; Immunotherapy; Dendritic cells; Cytokine induced killer cells; Progression-free survival; Overall survival.

Abbreviations AEs: Adverse Events; CBR: Clinical Benefit Rate; CIK: Cytokine Induced Killer Cells; CR: Complete Response; DCs: Dendritic Cells; MM: Malignant Melanoma; ORR: Objective Response Rate; OS: Overall Survival; PBMCs: Peripheral Blood Mononuclear Cells; PFS: Progression-Free Survival; PR: Partial Response; SD: Stable Disease

Introduction

Malignant Melanoma (MM) is one of the most malignant tumors, and its incidence is gradually increasing in recent years [1]. Metastatic MM has a very poor prognosis with a median survival time of 6-8 months and a 5-year survival rate of 6% [2,3]. Since advanced MM is not sensitive to conventional chemotherapeutic agents, great efforts have been made in the investigation of new drugs, such as tyrosine kinase inhibitors and immune checkpoint blockades. Although the new therapeutic agents have improved progression-free and Overall Survival (OS) of patients with MM, their use is limited by drug resistance and drug-related toxicity [4-8].

At present, immunotherapy has become the fourth most common therapy for solid tumors following surgery, chemotherapy, and radiotherapy [9,10]. In 1991, Schmidt-Wolf observed a novel type of antitumor effector cell, which was termed Cytokine-Induced Killer (CIK) cell [9]. CIK cells are a heterogeneous cell population, which possess major Histocompatibility Complex (MHC)-unrestricted cytotoxicity towards solid tumor by secreting a number of cytokines and chemokines [11-14]. CIK cells proliferate rapidly *in vitro*, and possess strong antitumor activity against a broad spectrum of solid tumors [15]. A number of clinical trials have reported that treating patients with a variety of solid tumors using CIK cells significantly improves the median survival time and may improve the immune function of patients [15-19].

Dendritic Cells (DCs) have been shown to be the most powerful antigen presenting cells, which can promote the production of cytotoxic T lymphocytes and helper T lymphocytes and participate in the immune response of the body. In addition, co-culture of DCs and CIK cells could enhance the antitumor activity of CIK cells without an increase of adverse effects [20]. Earlier clinical studies showed that DCs-CIK immunotherapy was a safe and effective treatment for patients with malignant tumors, such as non-small cell lung carcinoma, hepatocellular carcinoma and renal cell carcinoma [21,22]. In general, the immunogenicity of melanoma is higher than non-small cell lung carcinoma, etc., and we have reason to believe that DCs-CIK immunotherapy will benefit the melanoma patients. In this study, we performed a retrospective study to evaluate the efficacy and safety of

using DCs-CIK cells as an adjuvant therapy for advanced MM patients after conventional therapy.

Materials and Methods

Patients cohort and selection criteria

The inclusion criteria were as follows: the melanoma was confirmed by pathological examination, with no previous treatment, Karnofsky Performance Score (KPS)>60, and expected survival time longer than 6 months. The exclusion criteria were as follows: had history of previous immune therapy, with other previous or simultaneous malignant tumor, previous cancer treatment, allergic disorder, pregnant or breast-feeding women, and has been included in other clinical studies. All participants obtained verbal and wrote informed consent before enrolment.

Variable	DCs-CIK group (n=10)	Control group (n=12)	P-value
Sex, N (%)			
Male	5 (50.0)	4 (33.3)	0.666
Female	5 (50.0)	8 (66.7)	
Age, median, y	63	51.5	0.165
Treatment modality, N (%)			
Surgical resection	6 (60.0)	7 (58.3)	0.95
chemotherapy	5 (50.0)	8 (66.7)	
radiotherapy	3 (30.0)	3 (25.0)	
IFN	3 (30.0)	3 (25.0)	
TNM Stage, N (%)			
III	1 (10.0)	3 (25.0)	0.595
IV	9 (90.0)	9 (75.0)	
Clark Grade, N (%)			
3 grade	7 (70.0)	9 (75.0)	0.69
4 grade	2 (20.0)	1 (8.3)	
5 grade	1 (10.0)	2 (16.7)	
Depth of tumor, N (%)			
1 mm	1 (10.0)	0	0.235
1.01-2 mm	0	2 (16.7)	
2.01-4 mm	0	0	
4 mm	9 (90.0)	10 (83.3)	
Ulcer, N (%)			
Yes	3 (30.0)	7 (58.3)	0.675
No	7 (70.0)	5 (41.7)	

Table 1: Patients baseline demographics and disease characteristics

From August 2013 to August 2015, 22 advanced MM patients hospitalized in the First Affiliated Hospital of Third Military University, conforming to the inclusion criteria, were enrolled in this study, with 10 patients received DCs-CIK immunotherapy after surgical resection, chemotherapy or radiotherapy (DCs-CIK group), and 12 patients who did not receive DCs-CIK immunotherapy after conventional therapy (control group).

Preparation and treatment of DCs-CIK cells

Peripheral Blood Mononuclear Cells (PBMCs) were isolated from blood of patients by a Ficoll-Hypaque gradient centrifugation, and then cells were adhered in six-well plates for 2 h with a density of 3×10^6/mL in GT-T561 medium (TaKaRa, Inc) containing 5% self-serum.

Total count × 10^9	DCs-CIK group (n=10)
Mean ± SD	3.27 ± 0.8
Range	1.5-7.0
Cell viability %	
Mean ± SD	97.8 ± 1.6
Range	93-100
CD3+ cell %	
Mean ± SD	96.6 ± 2.2
Range	89.2-99.2
CD4+ cell %	
Mean ± SD	18.2 ± 7.5
Range	6.3-38.7
CD8+ cell %	
Mean ± SD	80.5 ± 6.2
Range	63.2-90.8
CD56+ cell %	
Mean ± SD	23.7 ± 7.3
Range	12.3-39.6
Injection cycles	
1-3	3
4-7	4
8-11	3
Total	**47**

Table 2: General condition of injected DCs-CIK cells of patients.

To generate DCs, adherent cells were incubated in 2 mL GT-T561 medium containing 5% self-serum, 1000 U/mL recombinant human granulocyte macrophage colony (Peptrotech, Inc), 1000 U/mL recombinant human interleukin-4 (Peptrotech, Inc), 100 U/mL penicillin, and 100 μg/mL streptomycin. After being cultured for 4 days, DCs maturation was achieved by adding 1000 U/mL tumor

necrosis factor-α, 10 ng/mL IL-1β, 10 ng/mL IL-6, and 1 μg/mL PGE2, and then they were cultured for another 3 days.

The method for the generation of CIK cells was slightly modified from previous methods [23]. Briefly, 1000 U/mL human recombinant IFN-γ (Peptrotech, Inc) was added to PBMCs on culture day 0. After 24 h of incubation, 50 ng/mL antibody against CD3 (Miltenyi Biotec, Inc), 100 U/mL IL-1α (Invitrogen, Inc) and 300 U/mL IL-2 (Peprotech, Inc) were added. Cells with a density of 3×10^6/mL were sub-cultured every 2-3 days in fresh complete medium containing 300 U/mL IL-2. The cells were co-cultured with DCs on culture day 7, and then cultured until 14 days to obtain DCs-CIK cells.

DCs-CIK cells treatment

Patients received DCs-CIK cells at each cycle, with number of cells ranging $(2.6\text{-}8.3) \times 10^9$. The rute/mode of administration for DCs-CIK cells was intravenous drip. Patients received at average 4.7 cycles of DCs-CIK cells transfusion, and the interval of every cycle was 2 weeks. The patients were eligible for maintenance treatment if they were disease-stable.

Efficacy	DCs-CIK group (n=10)	Control group (n=12)
CR	0	0
PR	2 (20.0%)	2 (16.7%)
SD	6 (60.0%)	3 (25.0%)
Progression	2 (20.0%)	7 (58.3%)
ORR	2 (20.0%)	2 (16.7%)
CBR	8 (80.0%)	5 (41.7%)
CR: Complete Response; PR: Partial Response; SD: Stable Disease; ORR: Objective Response Rate; CBR: Clinical Benefit Rate		

Table 3: Short-term clinical efficacy of patients.

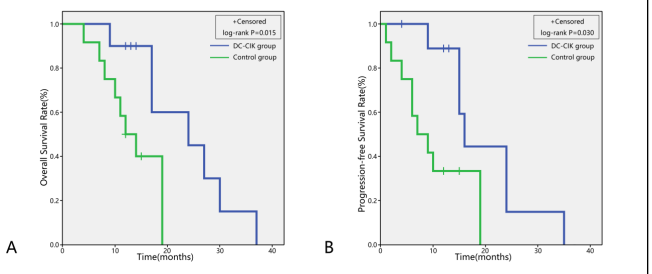

Figure 1: Kaplan-Meier estimates of overall survival (OS) and progression-free survival (PFS) of malignant melanoma patients treated with conventional therapy and DCs-CIK immunotherapy (CIK group, n=10) or conventional therapy alone (control group, n=12). (A) OS curves for DC-CIK versus control group (P=0.015). (B) PFS curves for DC-CIK versus control group (P=0.030).

Follow-up

All patients were followed up regularly. The follow-up included clinical and laboratory examinations or phone-call inquiring every 3 months until August 31, 2016 or the patient died. The median follow-up time was 17.0 months. The efficacy was assessed according to Response Evaluation Criteria in Solid Tumors (RECIST), version 1.1. Adverse Events (AEs) were classified and graded according to the Common Terminology Criteria for Adverse Events, version 3.0. The efficacy and AEs were assessed from the time the patient provided written informed consent to at least 30 days after the last immunotherapy.

Statistical analysis

The primary endpoints included OS and Progression-Free Survival (PFS). OS was measured from the date of enrolled until deathand PFS was measured from the date of enrolled to the first recurrence or death. The second endpoints included Objective Response Rate (ORR), Clinical Benefit Rate (CBR), and safety profile. OS and PFS were assessed by Kaplan-Meier curves using log-rank test. Unadjusted Hazard Ratios (HRs) were estimated using the Cox proportional hazards model. A cox proportional hazard analysis was performed to assess the effect of baseline characteristics on each outcome of interest. All statistical analysis was performed by SPSS version 13.0 for windows. The product and licence details for SPSS version 13.0 is 55345 26752 51345 33006 86803 93573 71895 65752 87. $P<0.05$ was considered as significant.

Outcome	DCs-CIK group (n=10)	Control group (n=12)	P value
PFS %			
12 months	88.9	25.0	
24 months	25.0	0	
PFS mo			
Median	16.0	7.0	0.030
OS %			
12months	90.0	50.0	
24 months	45.0	0	
OS mo			
Median	24.0	12.0	0.015

Table 4: Efficacy measures of patients.

Results

General description

There were no statistically significant differences in the baseline characteristics, including sex, age, treatment modality, TNM stage, Clark grade, Blesrow, and ulcer of patients between the two groups (Table 1). Patients in DCs-CIK group received DCs-CIK cells containing an average of 3.27×10^9 cells at each cycle (Table 2).

Analysis of disease-free survival and overall survival

Entire patient cohort: The short-term clinical effects were complete response (CR) 0%, Partial Response (PR) 20%, Stable Disease (SD) 60%, Objective Response Rate (ORR: CR+PR) 20%, Clinical Benefit

Rate (CBR: CR+PR+SD) 80% in DCs-CIK group, and CR 0%, PR 16.7%, SD 25%, ORR (CR+PR) 16.7%, CBP (CR+PR+SD) 41.7% in control group (Table 3).

OS and PFS were assessed in all 22 eligible patients. The 1 and 2 year OS rates of patients in DCs-CIK group and control group were 90.0%, 45.0% and 50.0%, 0, respectively. The median OS of patients in DCs-CIK group (24 months) was 12 months longer than that in control group (12 months). The difference in OS between the two groups was statistically significant (P=0.015), and patients in DCs-CIK group exhibited a better prognosis than that in control group (Figure 1A and Table 4). The univariate Cox proportional hazards regression analysis showed that ulcer and the treatment method were prognostic factors for OS in MM patients. On multivariate analysis, ulcer and adjuvant DCs-CIK immunotherapy were proven to be the independent prognostic factors for OS (Table 5).

The 1 and 2 year PFS rates of patients in DCs-CIK group and control group were 88.9%, 25.0% and 25.0%, 0, respectively. The median PFS of patients in DCs-CIK group (16.0 months) was 9.0 months longer than that in control group (7 months). The difference in PFS between the two groups was statistically significant (P=0.030) (Figure 1B and Table 4). The univariate Cox proportional hazards regression analysis showed that ulcer and the treatment method were prognostic factors for PFS in MM patients. Multivariate survival analysis showed that ulcer and DCs-CIK cell immunotherapy were the independent prognosis factors for PFS (Table 5).

	OS				PFS			
	Univariate analysis		Multivariate analysis		Univariate analysis		Multivariate analysis	
Variables	HR (95% CI)	P	HR (95% CI)	P	HR (95% CI)	P	HR (95% CI)	P
Age ≥ 59 *vs.* <59 yrs	1.176 (0.420-3.298)	0.758			0.932 (0.333-2.611)	0.893		
Sex male *vs.* female	1.328 (0.470-3.747)	0.592			1.007 (0.355-0.859)	0.990		
Clark grade 5 grade, 4 grade *vs.* 3 grade	1.042 (0.514-2.113)	0.908			0.978 (0.482-0.985)	0.951		
Blesrow <1 mm, 1.01-2 mm, 2.01-4 mm, >4 mm	1.168 (0.565-2.416)	0.675			1.059 (0.531-2.111)	0.871		
TNM stage III stage *vs.* IV stage	0.549 (0.147-2.043)	0.371			0.281 (0.069-1.155)	0.078		
Ulcer yes vs. no	4.163 (1.052-6.481)	0.042*	17.634 (2.909-06.898)	0.002*	2.932 (2.839-10.247)	0.092+	10.596 (2.237-0.196)	0.003*
Treatment group DCs-CIK group *vs.* control group	4.419 (1.167-6.737)	0.029	7.220 (1.412-36.907)	0.018*	3.460 (1.036-11.559)	0.044	6.672 (1.553-8.673)	0.011*

OS: Overall Survival; PFS: Progression-Free Survival; *P<0.05 For further correction of ulcer factor, we included it in the multivariate analysis model of both OS and PFS

Table 5: Univariate and multivariate analysis of factors associated with OS and PFS

Further analysis based on ulcer stratification

We performed subgroup analysis to determine whether ulcer affected the prognosis of MM patients, and the results showed that the 1 and 2 year OS rates for ulcer-positive patients in DCs-CIK group were 66.7% and 33.3%, in control group were 42.9% and 0, respectively. The 1 and 2 year PFS rates for ulcer-positive patients in DCs-CIK group were 33.3% and 33.3%, in control group were 28.6% and 0, respectively. A log-rank analysis showed the difference in OS and PFS between the two groups were not statistically significant, though ulcer-positive patients in DCs-CIK group had higher OS and PFS rates than those in control group (POS=0.072, PPFS=0.072) (Figures 2A and B).

Conversely, the 1 and 2 year OS rates of ulcer-negative patients in DCs-CIK group were 85.7% and 28.6%, respectively, and in control group were 80.0% and 0, respectively. The 1 and 2 year PFS rates of ulcer-negative patients in DCs-CIK group were 83.3% and 16.7%, respectively, and in control group were 20.0% and 0, respectively. A log-rank analysis showed that there were significant differences both in OS and PFS of ulcer-negative patients in the two groups (POS=0.037, PPFS=0.023) (Figures 2C and D).

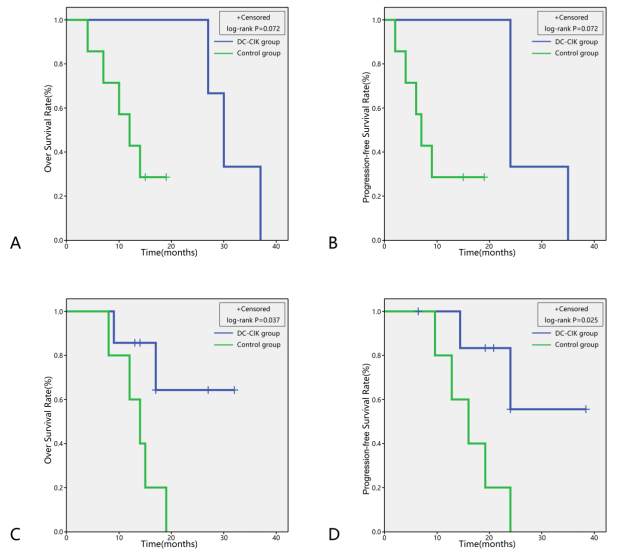

Figure 2: Kaplan-Meier estimates of overall survival (OS) and progression-free survival (PFS) of malignant melanoma patients treated with conventional therapy and DCs-CIK immunotherapy (CIK group) or conventional therapy alone (control group) and further grouped by ulcer status. The survival of ulcer-positive patients in the DC-CIK group (n=7) and the control group (n=5) was analyzed according to (A) OS curves (P=0.072), or (B) PFS curves (P=0.072). The survival of ulcer-negative patients in the DC-CIK group (n=3) and the control group (n=7) was analyzed according to (C) OS curves (P=0.037), or PFS curves (P=0.023).

Adverse effects of DCs-CIK cells therapy

No serious adverse events were observed in this study. Several mild adverse events were observed, which rapidly resolved without treatment (Table 6).

Adverse events	DCs-CIK group (n=10)	
	Any grade	Grade 3 or 4
Overall incidence	4 (40%)	
Fatigue	2 (20%)	0
Pyrexia	0	0
Myalgia	2 (20%)	0
Headache	0	0
Chill	0	0

Table 6: Adverse events of patients.

Discussion

Advanced MM has a very poor prognosis, and chemotherapy is the most important treatment for advanced MM patients. In recent years, immunotherapy has been widely used in clinic, and has achieved encouraging results. In this study, we investigated the efficacy of DCs-CIK immunotherapy for the advanced MM patients after conventional therapy. To the best of our knowledge, this is the first study to analyze the efficacy and safety of using DCs-CIK immunotherapy in human advanced MM. In total survival analysis, patients in DCs-CIK group had very significant improved 1, 2 year OS and PFS compared with control group. ORR and CBR were also improved in patients who received DCs-CIK immunotherapy compared with who not. These results indicated that the DCs-CIK immunotherapy as an adjuvant therapy played an active role in prolonging the survival of MM patients after conventional therapy. Simultaneously, multivariate survival analysis showed that the DCs-CIK immunotherapy was an independent prognostic factor for PFS of MM patients, indicating that DCs-CIK cells transfusion could be an effective therapy to improve the outcome of MM patients.

In this study, we also pay attention to ulcer. The results demonstrated that, for patients with advanced MM, adjuvant DCs-CIK therapy was only beneficial for ulcer-negative patients, and had no statistically significant benefit for ulcer-positive patients. Indeed, as shown in Table 5, ulcer was identified as an independent risk factor for OS and PFS of MM patients in our study. Other studies have also found that ulcer was closely associated with poor outcome of patients with MM [24]. In addition, ulcer-positive patients with MM have deeper invasion and larger damage on the body than ulcer-negative patients, and often accompany with lymph nodes and distant metastasis. So ulcer positive patients with MM mostly have poor prognosis [25]. Likewise, in our study, there was a total of 10 patients with ulcer (3 in DCs-CIK group and 7 in control group), and they suffered recurrence or metastasis, 50% of which occurred within 1 year (1/3 in DCs-CIK group and 4/7 in control group). Moreover, in our study, 9 patients suffered recurrence or metastasis. Among them, ulcer-positive patients accounted for 55.6% (5/9 patients), which was higher than that of ulcer-negative patients (4/9 patients). The high risk of recurrence or metastasis suggests that ulcer-positive patients possess a higher tumor burden than ulcer-negative patients. As an immunotherapeutic modality, Linn et al. [26] thought CIK transfusion may be more effective in patients with a lower tumor burden. Jiang et al. [27] reported that after partly clearing of tumor by PFS resection or other treatment, appropriate adjunctive immunotherapy could remove the minimal residual lesions and repair damaged immune function. In line with these suggestions, DCs-CIK immunotherapy did not seem to be beneficial to the ulcer-positive patients in our cohort. However, in our study, OS and PFS of patients with ulcer in DCs-CIK group were slightly higher than that in control group. That may be because of the frequency of follow-up of patients in DCs-CIK group, which only included three patients, was higher than that in control group. Thus, we are ready to begin prospective studies with large cohort scale.

It is known that T-cell-mediated immune responses play a significant role in antitumor activity [28]. In our study, patients in DCs-CIK group received transfusion of optimized and viable DCs-CIK cell populations, which approximately contained (96.6 ± 2.2)% $CD3^+$ cells, (77.1 ± 6.2)% $CD3^+/CD8^+$ cells, and (23.7 ± 7.3)% $CD3^+/CD56^+$ cells. What's more, the CD3+/CD56+ subset of NK-like T cells is unique, as it is a more terminally differentiated, late-effector T-cells population that possesses stronger cytotoxicity and a higher proportion of $CD8^+$ cells than the $CD3^+$/CD56- subset. $CD3^+/CD56^+$ T cells exert more potent antitumor toxicity than $CD3^+$/CD56- T cells *in vitro* studies [29,30]. An earlier report showed that CIK cells infusion not only killed the residual tumor cells directly but also improved the immunological status of MM patients through the

production of inflammatory cytokines. CIK cells transfusion could change the ratio of T lymphocytes in peripheral blood of patients, thus result in attenuated immune suppression and enhanced immune system's tumor clearance ability [31]. Though negative results have been reported on 13 Acute Myelogenous Leukemia (AML) patients who received autologous CIK, and survival and relapse did not change in the AML group with respect to controls [32]. Several studies indicated that co-culture of CIK cells with DCs led more proliferative activity than homologous CIK cells and more cytotoxic activity against tumor than CIK cells without co-culture. The secretion of IL-2, IL-12, IFN- and other cytokines would increase through the co-culture of CIK cells and DCs, in turn, cytokines can speed up the proliferation rate of CIK cells [33]. DCs secrete a large number of IL-12, which could promote CIK cells to highly express CD56+, and could further enhance the cell toxicity and anti-tumor activity. Furthermore, DCs could inhibit the activity of regulatory T lymphocytes to a certain extent, thereby enhance the killing activity of CIK cells [34]. This suggested that adjuvant DCs-CIK immunotherapy could have more of an impact on immunity and tumor killing compared with CIK therapy only in MM patients. Furthermore, for MM patients with solitary tumor and negative ulcer, the tumor would most likely to be eradicated after conventional therapy and with minimal residual lesions, which make them as ideal candidates for DCs-CIK immunotherapy.

Indeed, DCs-CIK immunotherapy is not completely without toxicity. However, studies from our institution and other institutions have confirmed its safety [22]. There were some limitations in the current study. First, the cohort scale is not large enough, especially the number of patients in DCs-CIK group. Second, the patients are not randomly assigned to each group, which may lead to potential selection bias. Third, this study was conducted at a single center. Therefore, a multicenter randomized controlled trail with large scale is needed to verify these findings.

In summary, from this retrospective study, we provide evidence that DCs-CIK immunotherapy is safe and could effectively improve the prognosis of melanoma patients after conventional therapy, especially melanoma patients with ulcer-negative.

Conflict of Interest

The authors declare that they have no conflict of interest.

Ethical Approval and Ethical Standards

The study protocol and informed-consent forms were reviewed and approved by the First Affiliated Hospital of Army Medical University ethical committee. All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. All individual participants included in the study must give informed consent.

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