RESEARCH ARTICLE

Impact of Cellular Immune Function on Prognosis of Lung Cancer Patients after Cytokine-induced Killer Cell Therapy

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Abstract

Aims: To investigate changes in cellular immune function of patients with lung cancer before and after cytokine-induced killer (CIK) cell therapy and to identify variation effects on overall survival (OS) and progression-free survival (PFS). Materials and Methods: A total of 943 lung cancer patients with immune dysfunction were recruited from January 2002 to January 2010, 532 being allocated to conventional therapy and 411 to CIK therapy after a standard treatment according to the NCCN Clinical Practice Guidelines. All the patients were investigated for cellular immune function before and after therapy every three months. and clinical prognostic outcomes were analyzed. Results: After six courses of treatment, immune function was much improved in patients receiving CIK cells therapy as compared to controls. The percentages of recurrence and/or metastases for patients undergoing CIK cell therapy was 56.2% and 49.1% respectively but 78.6% and 70.3% among controls (p<0.001). The median OS times for CIK cell therapy and control groups were 48 and 36 months respectively. The OS rates at 12, 36, 60, 84 months in CIK treated patients were 97.8%, 66.9%, 27.7%, and 4.1% while they were 92.3%, 44.5%, 9.2%, and 1.5% in controls. OS and PFS were significantly different by log rank test between the two groups and across the three immune improvement classes. Conclusions: The immune function of lung cancer patients was improved by CIK cell therapy, associated with an increase in the OS rate and extension of the time to recurrence and/or metastasis.

Keywords: CIK cell - lung cancer - prognosis - immune function

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Introduction

Lung cancer is the leading cause of cancer death for each sex in both developed and developing countries. Globocan 2008 estimated that lung cancer was the most diagnosed cancer and the leading cause of cancer death among men and was the fourth commonest form of cancer and the second leading cause of death among women (Jemal et al., 2011). Among an estimate of 12.7 million new cancer cases and 7.6 million cancer death in 2008, lung cancer accounted for 13% (1.6 million) of the total cases and 18% (1.8 million) of the deaths.

The "Chinese Cancer Registry Annual Report 2012" estimated that the age standardized incidence rate (world standard population) for all cancer cases and death rate were 191.7 and 115.6 per 100,000 population while the age standardized rates for 1982 Chinese population (ASRIC for incidence and ASRMC for mortality rates) were 146.9 and 85.1 per 100,000 population, respectively. Lung cancer was the most common cancer in with an ASRIC of 25.3 and 23.2 per 100,000 populations among males and females respectively (Chen, 2013). The incidence of lung cancer among Chinese females was the second most common cancer after breast cancer. It was the leading cause of death among both sexes with an ASRMC of 20.6 and 12.6 per 100,000 populations among males and females respectively (Chen, 2013).

Cancer immunotherapy using specificity of the immune system to cancer antigens is a novel treatment of malignancy. Although cancer cells are less immunogenic than pathogens, the immune system is clearly capable of recognizing and eliminating them (Blattman and Greenberg, 2004). Cytokine-induced killer (CIK) cells are recognized as newly identified anti-tumor effector cells that can proliferate rapidly *in vitro* with a stronger anti-tumor activity and a broader spectrum of targeted tumors than other reported anti-tumor effector cells of the same kind (Schmidt-Wolf, 1991; Hontscha et al., 2011). Moreover, CIK cells are found to be able to regulate and generally enhance immune functions in cancer patients (Schmidt-Wolf, 1991; Schmidt-Wolf et al., 1993). Treatment with CIK is a promising and safe

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modality for malignant neoplasms. Multicenter clinical trials were warranted to establish the validity of this therapeutic approach and optimize the CIK treatment protocol. These studies demonstrated that chemotherapy plus CIK cells have significant benefits for patients who suffered from advanced gastric or lung cancers with no severe side-effects (Schmidt-Wolf et al., 1999; Jiang et al., 2006; Wu et al., 2008; Olioso et al., 2009; Jiang et al., 2010). Therefore, CIK therapy was a new adoptive immunotherapy strategy developed in recent years for cancer treatment (Blattman and Greenberg, 2004).

Nowadays surgery is the first choice and effective treatment for early lung cancer patients. However, most of them are diagnosed at advanced stage or metastasis and lost opportunity of surgery. With the advances in medical technology, immunotherapy becomes an entirely new modality of treatment for lung cancer patients in complement to chemotherapy and radiotherapy (Dougan and Dranoff, 2009; Stroncek et al., 2010; Hontscha et al., 2011).

Adoptive immunity is the extracorporeal modification process of immunological activity of a host after the immunological components are withdrawn from and then reinfused back into the same host. The adoptive transfer of autologous immunological components has been successful in treatment of patients with solid tumor (Zheng et al., 2013). The treatment enhanced the innate function of immune system to react against cancer (Schmidt-Wolf et al., 1993) and proven to be very effective in destroying the tumors in humans to achieved therapeutic effect.

Based on the aforementioned immunological basis, this study aimed to monitor the cellular immune function of lung cancer patients before and after CIK cell therapy, and to evaluate the clinical efficacy of CIK cell treatment in patients with lung cancer and patients from the clinical benefit of CIK cell treatment.

Materials and Methods

Study subjects

A total of 943 lung cancer patients were consecutively recruited from Yunnan Tumor Hospital at the out-patient and in-patient departments from January 2002 to January 2010 and were re-diagnosed according to the National Comprehensive Cancer Network (NCCN) Clinical Practice Guidelines (Sun et al., 2011). All of them had completed investigation for clinical staging according to American Joint Committee on Cancer (AJCC), obtaining cytology and/or biopsy for pathological typing. Patients with stage I, II and III non-small cell lung cancer (NSCLC) were recruited and those met the following conditions were excluded: (1) consolidated immune system disease; (2) complicated with chronic wasting disease and infectious diseases; or (3) combined with other malignancies. All eligible subjects were randomly allocated into two groups stratified by age group and sex by simple random number generation, one received CIK cell therapy for 18 months (6 courses) and another got conventional treatment.

This study was approved by the Ethical Committee of Yunnan Tumor Hospital, and printed informed consents were read, understood, and signed by all the participants.

Apparatus and reagents

Patients' blood was processed with CS-3000PLUS blood cell separator (Baxter International Inc., California, USA) Other instruments included Forma311 carbon dioxide incubator (Thermo Fisher Scientific, USA), Forma205 biological safety cabinet (Thermo Fisher Scientific, USA), serum-free medium (AMV) of Gibco Company (USA), and Epics XL flow cytometer (BECKMAN COULTER company, USA). The reagents used in the flow cytometry, CD45-FITC/CD4-RD1/CD8-ECD/CD3-PC5 and CD3-FITC/CD16+56-PE+CD45-PC5 were both purchased from IMMUNOTECH Company (France).

Treatment

After completed investigation of histopathology and stage, patients were allocated to the two arms of treatment protocol by simple random allocation. Patients received surgery and chemotherapy or radiotherapy based on their stage of disease according to the NCCN Clinical Practice Guidelines. Patients allocated to the conventional therapy arm were given with standard chemotherapy after surgery. Those allocated to CIK cells therapy received the standard chemotherapy for one month, and then underwent autologous immune cells *in vitro* amplification and reinfusion. The process of cell preparation and reinfusion are described below.

CIK cells preparation

The peripheral blood mononuclear cells (PBMC) of patients were collected via blood cell separator and suspended in serum-free medium. After adjusting the cell density to 1×10^6/mL, then the cells were seeded in culture flasks at 37 °C in 5% CO_2 incubator for culture. At hour 0 1,000 U/mL γ-IFN were added. After 24 hours of incubation, 300 U/mL IL-2 and 50 ng/mL CD3McAb were added and the culture was continued. The medium was replaced every 4 days and the cell density was adjusted to 1×10^6/mL with 300 U/mL IL-2. The IL-2 and 50 ng/mL of CD3McAb were refilled every 8 days. The cells were then collected in a week later.

CIK cells reinfusion at the end of cell culture, with negative microbial test , the culture solution was washed three times with saline, and the cell suspension in the liquid compound made of 20% human serum albumin 30 mL and 100 mL saline was ready for the venous reinfusion once a day for 3 consecutive days as a course of treatment. The number of cells in each reinfusion was around $2 \sim 6\times10^9$ cells/mL.

Assessment of immune function

We defined the patient whose cellular immune function condition was unchanged either in the group receiving conventional treatment or CIK cells therapy for 6 courses as no improvement (NI).

The patient whose cellular immune function had shown to be improved at any stages but unable to be maintained as long as six months at the end of conventional treatment or at the completion of 6 courses of CIK cells treatment was considered as short-term improvement (SI).

Those whose cellular immune function had

improvement at any times and normal immune function could be maintained at the end of six months of conventional treatment or CIK cells therapy was documented as continuous improvement (CI).

Statistical analysis

Data analysis was conducted using SPSS software (version 13.0). The t-test and Chi-square test were used for comparing of continuous and categorical variables respectively. Normality of distribution and equality of variance tests were done before applying a t-test. Distributions of survival time and survival proportion were determined by the Kaplan–Meier method. The survival proportion at 1, 3, 5, 7 years were reported. Overall survival (OS) was computed as the time between the first day of treatment and the date of death or the last day on which patient was known to be alive. Progression free survival (PFS) was calculated based on the date of initiation of treatment to the date of disease progression or death. Statistical significance between two survival proportions was assessed by using the log-rank test. P values were considered significant at less than 0.05 (two tailed). Ninety five percent confidence interval was calculated to depict the possible range of the estimates where appropriate.

Results

Patient characteristics in both CIK cell therapy and conventional treatment groups were displayed in Table 1. No statistical significant difference was found between the two groups for the general clinical variables of age, sex, clinical stage, pathological category, cellular immune function disorder, Karnofsky performance status (KPS) grade, and so on.

Table 2 displays the distribution of the three immune function improvement categories among the two groups by different clinical statuses, all patients, patients with disease recurrence, and patients with metastasis. Only 30.2% of lung cancer patients' immune function did not improve after CIK cells therapy for six courses while 71.6% of patients without CIK therapy showed no improvement (p value <0.001). After six courses of CIK cells therapy, the patient immune function with SI and CI accounted for 47.7% (95% CI: 42.8-52.6) and 22.1% (95% CI: 18.2-26.5), respectively, while the percentage of SI and CI in control group was 19.7% (95% CI: 16.4-23.4) and 8.6% (95% CI: 6.4-11.4), respectively. The difference in the percentage in improvement status between the two groups was obviously significant.

The proportion of recurrence in lung cancer patients with standard treatment and with CIK cells therapy was statistically significant, 78.6% vs. 56.2%. It was also true for metastasis where the proportion in control group was 70.3% while it was 49.1% in CIK treatment group. The difference was also observed between the two treatment groups within the same improvement status where patients in CIK group exhibited less recurrence and metastasis with

Table 1. Comparison between Control and CIK Therapy Groups in Demographic and Clinical/ Pathological Information

	Conventional treatment group (n=532)		CIK therapy group (n=411)		p value*
Age, mean±SD	52.95±7.60		53.66±8.27		0.174
Gender, cases (percent)					
Male	378	(71.1)	276	(67.2)	0.198
Female	154	(28.9)	135	(32.8)	
Clinical stages, cases (percent)					
I+IIa	442	(83.1)	346	(84.2)	0.651
IIb+IIIa	90	(16.9)	65	(15.8)	
Pathological category, cases (percent)					
Squamous carcinoma	383	(72.0)	300	(73.0)	0.733
Adenocarcinoma	149	(28.0)	111	(27.0)	
Lymph node metastasis, cases (percent)					
Yes	30	(5.6)	25	(6.1)	0.773
No	502	(94.4)	386	(93.9)	
Preoperative chemotherapy, cases (percent)					
Yes	19	(3.6)	12	(2.9)	0.578
No	513	(96.4)	399	(97.1)	
Preoperative radiotherapy, cases (percent)					
Yes	26	(4.9)	24	(5.8)	0.518
No	506	(95.1)	387	(94.2)	
KPS grade, mean±SD	70.31±6.87		70.21±6.22		0.82
Cellular immune function, cases (percent)					0.845
CD4/CD8 normal					
CD3 decline	150	(28.2)	119	(29.0)	
CD4/CD8 decline					
CD3 normal	127	(23.9)	81	(19.7)	
CD3 decline	126	(23.7)	108	(26.3)	
CD3 rise	72	(13.5)	58	(24.8)	
CD4/CD8 rise					
CD3 normal	15	(2.8)	12	(2.9)	
CD3 decline	17	(3.2)	12	(2.9)	
CD3 rise	15	(4.7)	21	(5.1)	

*T-test p-values are for age and KPS grade, chi-square p-values are for categorical variables

Table 2. Improvement Status and Subsequent Recurrence and Metastasis in Control and CIK Cells Therapy Groups

Improvement status in the two groups	All recruited cases			Cases with subsequent recurrence			Cases with subsequent metastasis		
	N	%[a]	95%CI[a]	N	%[b]	95%CI[b]	N	%[b]	95%CI[b]
Control group	532	100		418	78.6		374	70.3	
NI	381	71.6	67.6–75.4	307	80.6	76.2–84.4	281	73.8	62.5-71.7
SI	105	19.7	16.4–23.4	82	78.1	69.0-85.6	69	65.7	55.8-74.7
CI	46	8.6	6.4–11.4	29	63	50.1-79.5	24	52.2	38.8-69.6
CIK therapy group	411	100		231	56.2		202	49.1	
NI	124	30.2	25.8–34.9	87	70.2	61.3-78.0	79	63.7	54.6-72.2
SI	196	47.7	42.8–52.6	108	55.1	47.9-62.2	95	48.5	41.3-55.7
CI	91	22.1	18.2–26.5	36	39.6	29.5-50.4	28	30.8	21.5-41.3

*NI=no improvement, SI=short-term improvement, CI=continuous improvement, 95%CI: 95% confidence interval; [a]percentage of cases in the control/CIK group; [b]percentage of cases in the same improvement group (NI, SI, or CI)

p-value <0.001 and the number of cases and the percentage with its 95% CI for each improvement status in the two treatment arms are given in Table 2.

Table 3 displays the metastasis and recurrence free survival proportion from 12 to 84 months in different cellular immune function status of both groups. The median survival time to recurrence and/or metastasis for lung cancer patients after CIK cells therapy was 42 months, and it was 32 months in control group. The metastasis/recurrence free survival rates after CIK cells therapy were 94.9%, 52.8%, 19.5%, and 1%while in control group they were 85.3%, 33.5% , 6%, and 0.9% at 12, 36, 60 and 84 months respectively. Log rank test p-values for the difference of survival between CIK treatment vs. control groups were 0.045, <0.001, and 0.172 for NI, SI and CI respectively. It is worth noting that the difference was not statistically significant in CI between the CIK treatment vs. control. Log rank test of difference in PFS between the two groups were statistical significantly different (p<0.001) (Figure 1).

Further analysis about the situation of PFS on different states of cellular immune function between the two groups of lung cancer patients, log rank test was used to see the difference of PFS for each state. There were statistically significant difference between NI and SI (log rank test p=0.008); between NI and CI (log rank test p<0.001); and between SI and CI (log rank test p<0.001) in control group. Similar results were detected in CIK cell therapy group (Figure 2 and 3) with log rank test p<0.001 in all comparisons.

Table 4 demonstrates the overall survival (OS) proportion and median survival time for various cellular immune functions after CIK cells therapy. The median OS time was 48 months in CIK cells therapy group and 36 months in control group. The OS proportions at 12, 36, 60 and 84 months in CIK cells therapy group were 97.8%, 66.9%, 27.7% and 4.1%, respectively, while in control group they were 92.3%, 44.5%, 9.2% and 1.5%,

Table 3. Progression-free Survival in Control and CIK Cells Therapy Groups Under Different Cellular Immune Function Status

	N	Metastasis and recurrence-free				Median survival time survival proportion	95%CI
		12 mo.	36 mo.	60 mo.	84 mo.	(months)	
Control group							
	532	0.853	0.335	0.06	0.009	32	31.2-32.8
NI	381	0.829	0.283	0.031	0	28	26.0-30.0
SI	105	0.886	0.343	0.067	0.01	33	31.3-34.7
CI	46	0.978	0.739	0.283	0.065	47	43.1-50.9
CIK therapy group							
	411	0.949	0.528	0.195	0.01	42	39.7-44.3
NI	124	0.903	0.315	0.032	0	32	29.5-34.5
SI	196	0.939	0.556	0.153	0.01	42	36.5-47.5
CI	91	0.989	0.758	0.505	0.022	61	57.2-64.8

*NI=no improvement; SI=short-term improvement; CI=continuous improvement; mo.=months; 95%CI: 95% confidence interval

Table 4. Overall Survival Proportion and Median Survival Time Under Different Cellular Immune Function Status in Control and CIK Cells Therapy Groups

	N	Overall survival proportion (months)				Median (months)	95%CI*
		12 mo.	36 mo.	60 mo.	84 mo.		
Control group							
	532	0.923	0.445	0.092	0.015	36	35.6-36.4
NI	381	0.906	0.367	0.047	0.005	33	31.4-34.6
SI	105	0.962	0.514	0.124	0.019	38	35.7-40.3
CI	46	0.978	0.935	0.391	0.087	58	52.0-64.0
CIK therapy group							
	411	0.978	0.669	0.277	0.041	48	44.7-51.2
NI	124	0.968	0.508	0.105	0.016	38	35.8-40.2
SI	196	0.995	0.673	0.25	0.041	48	42.5-53.5
CI	91	0.989	0.879	0.571	0.209	66	62.3-69.7

*95%CI: 95% confidence interval; **NI=no improvement; SI=short-term improvement; CI=continuous improvement; mo.=months

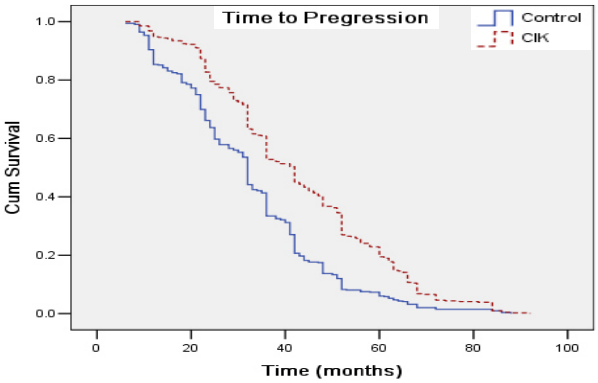

Figure 1. Progression-free Survival in the Control and CIK Cells Therapy Groups

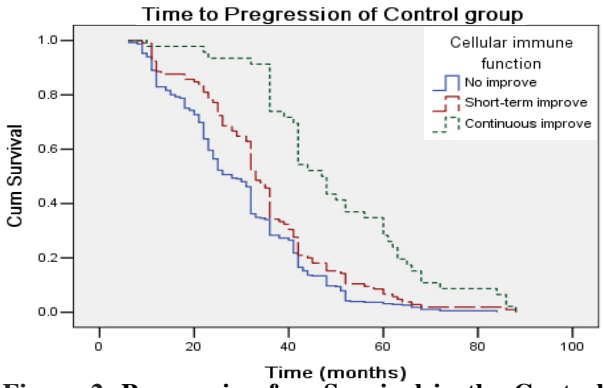

Figure 2. Progression-free Survival in the Control Group Under Different Cellular Immune Function Improvement Status

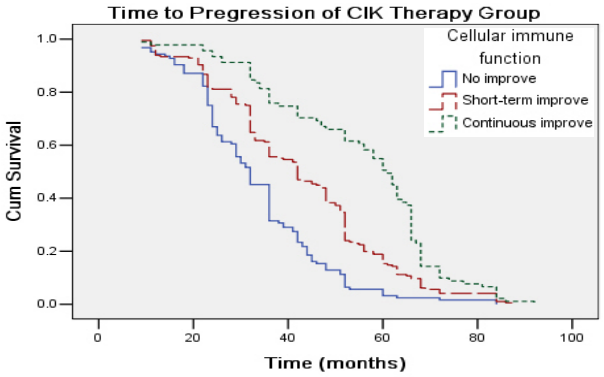

Figure 3. Progression Free Survival in the CIK Cells Therapy Group Under Different Cellular Immune Function Improvement Status

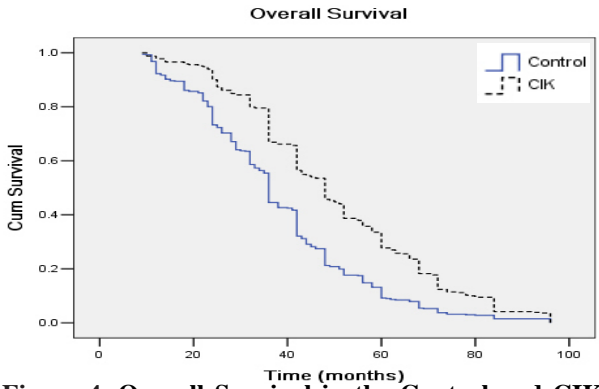

Figure 4. Overall Survival in the Control and CIK Cells Therapy Groups

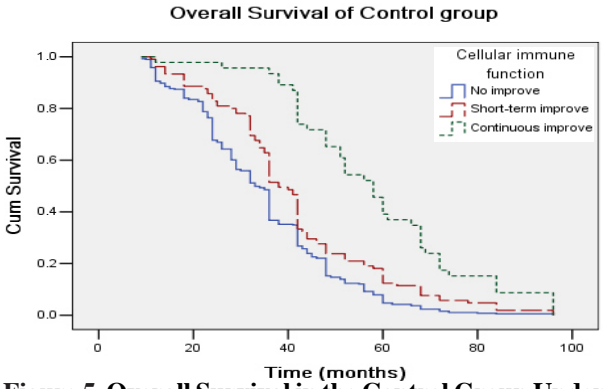

Figure 5. Overall Survival in the Control Group Under Different Cellular Immune Function Improvement Status

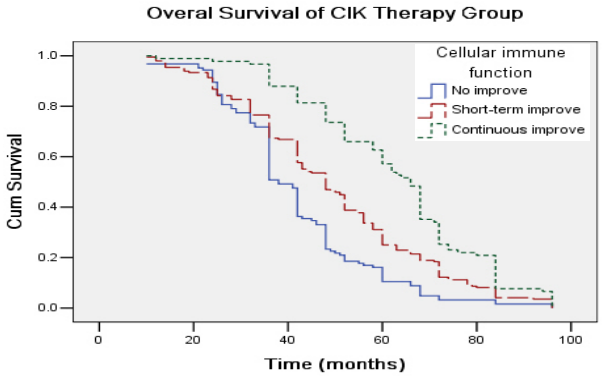

Figure 6. Overall Survival in the CIK Cells Therapy Group Under Different Cellular Immune Function Improvement Status

respectively. Log rank test p-values for the difference of OS between CIK treatment vs. control groups were <0.001, 0.001, and 0.265 for NI, SI and CI respectively. The indifference of OS between the two groups in CI was also present as it was of the recurrence and metastasis free survival. Log rank test for the difference between the two groups was significantly different (p<0.001) (Figure 4).

The OS rates under different situation of cellular immune function in the two groups of patients were statistically different in control group between NI and SI, between NI and CI, and between SI and CI, with log rank p values <0.001 in all comparisons. Similar results were also reproduced in CIK cell therapy group (Figure 5 and 6).

Discussion

In this study CIK cells therapy was superior to conventional treatment by enhancing the cellular immune function. It was consistent with other previous studies (Kim et al., 2007; Lin et al., 2010; Jin et al., 2013). The effects of CIK on prolonged PFS and OS had been demonstrated by other studies (Li et al., 2012; Zhong et al., 2013) and had been confirmed by this study.

The improvement in survival was considered as a criterion for evaluation of clinical benefit for patients in oncology. This study illustrated that the CIK cells therapy can improve both PFS and OS in patients with lung cancer, indicating CIK cells therapy had enhanced curative effect on the disease and the patients can achieve clinical benefit from it.

The results indicated that, compared with conventional therapy, a significant proportion of lung cancer patients after a course of CIK cells treatment had cellular immune function improved significantly, especially with the SI status. CIK cells treatment significantly reduced the recurrence and metastasis rates of patients and prolonged the median time of recurrence and metastasis. The metastases and recurrence rates from 1 to 5 years were reduced regardless of the patient with cellular immune function CI, SI or NI. CIK cells therapy prolonged median survival time. The overall survival rate at 1, 3, 5 year under the situation of cellular immune function variations was extended. However, it has to be accepted that long-term survival up to 7 years cannot be achieved by the CIK therapy so as the conventional treatment.

A study conducted in Shanghai of China reported that the patients could benefit from CIK cells therapy (Zhong et al., 2013). In that study on 60 patients with non-small-cell lung carcinoma (NSCLC) receiving four courses of navelbine-platinum (NP) chemotherapy, one group was treated with CIK cell transfusion and another group received immunotherapies. The 1-year and 3-year overall survival rates of patients receiving CIK cells therapy were 63.3% and 23.3%, and the rates of those receiving immunotherapies were 56.7% and 6.7%, respectively. The difference between the two groups was statistically significant (p=0.037) (Zhong et al., 2013). However, the OS regardless of treatment or control groups in that study was lower than that of our study. The possible explanation was that the patients' condition of present study was milder than that of Shanghai study.

Another study in Tianjin of China illustrated that NSCLC patients through CIK combined with chemotherapy compared with chemotherapy alone, 3-year median PFS was 39 and 32 months, respectively with a significant difference between the two groups (p=0.05). The median PFS were 34 and 17 months with a significant difference between the two groups (p=0.028). The results of that study confirmed the benefit of the CIK cells therapy in improving the effectiveness of conventional chemotherapy in NSCLC patients (Li et al., 2012). However, the progression-free survival in their study for patients either treatment group or the control group was obviously lower than the results in this study. One of the possible reasons might be similar to that mentioned above

that the patients selected in this study were mild compared with the two studies in Shanghai and Tianjin.

In conclusion, the above results suggest that patients with lung cancer through adjunct CIK cells adoptive immunotherapy compared with conventional therapy alone achieved good curative effect. It helps preventing cancer recurrence and metastases and also prolonged overall survival and progression-free survival in patients with lung cancer.

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