RESEARCH ARTICLE

# Adoptive Immunotherapy for Small Cell Lung Cancer by Expanded Activated Autologous Lymphocytes: a Retrospective Clinical Analysis

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## Abstract

**Background: To investigate the clinical efficacy of expanded activated autologous lymphocytes (EAAL) in patients with small cell lung cancer (SCLC). Materials and Methods: A total of 32 SCLC patients were selected and randomly divided into EAAL treatment and control groups, 16 cases in each. EAAL were obtained by proliferation of peripheral blood mononuclear cells (PBMCs) of patients followed by phenotype determination. Clinical data of all patients were recorded. Patients of both groups were followed up and the overall survival (OS) were compared retrospectively. Results: After culture and proliferation *in vitro*, the percentages of $CD3^+$, $CD3^+CD8^+$, $CD45RO^+$, $CD28^+$, $CD29^+$, $CD8^+CD28^+$ and CD3+CD16+/CD56+ cells increased markedly ($p<0.05$). The OS of the EAAL treatment group was longer than that of control group, but the difference was not statistically significant ($p=0.060$, HR=0.487, 95% CI 0.228~1.037). 1- to 3-year survival rates in EAAL treatment group were longer than those in control group, but there was still no significant difference ($p>0.05$). COX multivariate regression analysis showed that the number of chemotherapy cycles and the application of EAAL immunotherapy were independent prognostic factors for SCLC patients. The OS in females and chemotherapy≤6 cycles were obviously prolonged after EAAL immunotherapy. Conclusions: In vitro induction and proliferation of EAAL is easy and biologically safe. Generally, EAAL adoptive immunotherapy can evidently prolong the OS of SCLC patients.**

**Keywords:** Adoptive immunotherapy - small cell lung cancer - expanded activated autologous lymphocytes

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## Introduction

Small cell lung cancer (SCLC) is a highly malignant tumor with very poor prognosis. Most patients developed metastatic SCLC at diagnosis. For patients with SCLC, the median survival time (MST) is 8~12 months and the 5-year survival rate is about 2%. The staging of SCLC can be classified as limited stage and widespread stage. Even if in the limited stage, the MST of SCLC patients is only 18~20 months, and the 5-year survival rate is about 10% after treatment (Luo et al., 2012; Xiao et al., 2014). Chemotherapy is one of the primary therapeutic methods for SCLC. The etoposide (VP-16) plus cisplatin or paraplatin (EP or CE regimen) is the first-line standard chemotherapy for SCLC, which can achieve 60%~80% of objective response rate (ORR) and 20~40% of complete response rate (CRR). However, the effectiveness of the first-line chemotherapy lasts for a short time and the cancer progresses in most patients within 6 months after the discontinuation of chemotherapy. Relapse even occurs in some patients within 3 months after the withdrawal of the first-line treatment. The second-line treatment can benefit the survival of patients (Qian et al., 2014). The first-line maintenance treatment or the increased dosage is unable to obviously reduce the relapses and improve the survival rates. Instead, excessive dosage is worse than undone (Levy et al., 2013; Zhou et al., 2013). In recent years, molecular targeted therapy for SCLC has been extensively studied by many researchers. Unfortunately, targets for the occurrence of SCLC remain undefined and the results from clinical studies of investigational new drugs are not encouraging (Spigel et al., 2012). Therefore, the prognosis of patients with SCLC is not satisfying. Eventually, about 95% patients with SCLC died of progressive tumor.

Adoptive cellular immunotherapy has been considered as an important antitumor treatment for many years. Various in-vitro proliferated effector cells, such as lymphokine-activated killer (LAK) cells (Pitini et al., 2007; Kato et al., 2010), activated natural killer (NK) cells (Cho et al., 2010; Guo et al., 2010; Ahn et al., 2013; Saito et al., 2013), dendritic cells (DCs) ( Sabado et al., 2013; Ahmed et al., 2014), tumor-infiltrating lymphocytes

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(TILs) (Besser et al., 2010; Phan et al., 2013) and cytokine-induced killer (CIK) cells (Shi et al., 2013; Yang et al., 2013; Wang et al., 2014) have shown some anti-tumor effects. The expanded activated autologous lymphocyte (EAAL) is a new adoptive cellular immunotherapy that is developed to isolate the T lymphocytes from cancer patients with immobilized anti-CD3 monoclonal antibody. EAAL is proved to be a heterogeneous cell population containing about 30% CD4+ and 60% CD8+ cells (Sun et al., 2011). The results of a randomized clinical trial indicated that EAAL adoptive immunotherapy was a safe and feasible treatment that could improve the outcome of patients with hepatocellular carcinoma (HCC) by reducing the postoperative recurrence rates (Takayama et al., 2000). These findings demonstrated the superiority of EAAL over other immune cells used in adoptive immunotherapy. In addition to the decreased recurrence (18% in comparison with control groups), EAAL shows a mean expansion index of 1 560-folds (Takayama et al., 2000; Sun et al., 2011).

The potential benefit of EAAL in SCLC has not been explored. Therefore, this study aimed to examine the clinical effects of EAAL in a case-control study where the overall survival (OS) of SCLC patients was assessed.

## Materials and Methods

*In-vitro culture and proliferation of EAAL and testing of biological safety*

Activated lymphocytes using anti-CD3 monoclonal antibody (OKT3, eBioscience, Austria) and interleukin (IL)-2 were generated as described previously (Luo et al., 2012). Briefly, 20~100 mL of peripheral blood were collected from each patient and peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll-Hypaque gravity centrifugation. The isolated PBMCs were washed and re-suspended in serum-free medium IMSF100 (Immunotech, West Kensington, London) supplemented with 500 U/mL of IL-2. The PBMC suspension was then placed in a flask coated with immobilized anti-CD3 antibody and incubated for 1 week. The lymphocyte suspension was transferred to a gas permeable bag to grow for >2 weeks. The activated lymphocytes were then harvested and filtered through 100 μm membrane and re-suspended in 100 mL normal saline (NS) containing 1% human serum albumin for intravenous infusion. Before cell transplantation, the cells were tested for endotoxin levels using a Limulus Amebocyte Lysate kit (CAPECOD Incorporated, USA). Average cell count per 100 mL after the *in vitro* expansion was $(6.17\pm1.21)\times10^9$ for the patients who were then administrated with activated lymphocytes 100~200 mL (large dose) once a week. All of these activated lymphocytes for transfusion was cultured and detected in the lab of Immunotech Applied Sciences.

The cell count and viability before and after incubation was determined using trypan blue exclusion test. A small volume of cell suspension that was appropriately diluted and mixed thoroughly with trypan blue was taken. A bit of mixture was added to the blood counting chamber through the upper groove and the cover slip was used. Then the cells were observed under microscope. The viable cells were not stained while dead cells were stained with blue. The total cells and non-stained cells in 4 big boxes were counted respectively. The number of cells was calculated based on the following formula: The number of cells (cells/ML) = (The total number of cells in 4 big boxes/4)×10,000×dilution factor. Only the upper and left parts were counted. The total number of cells was determined based on the cell liquid volume before and after incubation. The number of non-stained cells was divided by the total number of cells and then multiplied by 100% to get the cell survival rate. The total number of cells after incubation was divided by the total number of cells before incubation to get the amplification factor.

*Phenotype detection of patients' peripheral blood lymphocytes and EAAL cells*

3~5 mL of fasting blood was collected via veins from each patient with SCLC on early morning using an EDTA-Na2 contained anticoagulant tube. 100~200 μL of blood containing EDTA- Na2 of each patient was added to 7 tubes for the perform of flow cytometry (Bechman-Coulter Company, USA) with pipettes (Eppendorf, Germany). Each blood sample was added with 10 μL of anti CD8 - FITC/CD4 - PE/CD3 - PC5, CD3 - FITC/CD56 - PE/CD16 - PC5, CD45RO - FITC, CD29 - FITC, CD8 - FITC, CD25 - PE, CD45RA - PE, CD28 - PE, CD19 - ECD and CD4 - PC7 fluorescent antibodies as well as negative isotype controls (Bechman - Coulter Company, USA) of the above antibodies. After thorough mixing, the samples were placed at room temperature for 20 min without exposure to the sunlight. Next, these tubes were placed in an automatic hemolysis instrument called Q-prep, and successively added with 600 μL solution A (0.12% formic acid), 265 μL solution B (containing 0.6% $Na_2CO_3$, 1.45% NaCl and 3.13% $Na_2SO_4$) and 100 μL solution C (1% formic acid). Then they were evenly mixed and shaken for 15 s. The treated samples were placed at room temperature for 5~10 min. After the red cells were completely broken in hemolytic condition, the samples were analyzed using flow cytometer. A total of $5\times10^5$ EAAL harvested cells were collected and washed twice with phosphate buffer solution (PBS) and re-suspended with 2 mL PBS. After that, 100~200 μL EAAL cell liquid was added to 7 tubes for flow cytometry with pipettes. Each cell sample was added with 10 μL of the above fluorescent antibodies as well as their negative isotype controls. After thorough mixing, the cell samples were placed at room temperature for 20 min without exposure to the sunlight. Then 1 mL PBS was added to re-suspend the cells and the cell samples were analyzed using flow cytometer.

*Patients and trail design*

A total of 32 SCLC patients were selected from the Department of Clinical Oncology, Chinese PLA General Hospital, in whom there were 19 males and 13 females, aged from 35~68 years with median age being 60 years. All patients were selected according to the *NCCN: Clinical Practice Guideline for Small Cell Lung Cancer (Version 2011)* and all signed the informed consent forms. This retrospective case control study was approved by the Medical Ethics Committee of PLA General Hospital.

First of all, patients who were pathologically diagnosed as SCLC and received EAAL treatment previously were selected based on the records of cell therapy. According to the medical records of the electronic medical records system of Chinese PLA General Hospital, patients with SCLC were assigned to EAAL treatment group after those with ECOG score>2, estimated survival<12 weeks, loss of follow-up or incomplete clinical data were excluded. According to the medical records and follow-up records, clinic data such as gender, age, date of hospitalization, clinical staging, surgery, radiotherapy, chemotherapy and OS were recorded for each patient in EAAL treatment group. Meanwhile, the frequency of EAAL treatment, the combination with surgery, radiotherapy or chemotherapy and EAAL treatment-related adverse reactions were recorded in detail. Similarly, patients who were pathologically diagnosed as SCLC and admitted to the hospital in the same month were also selected. Patients with SCLC were pooled as candidates of control group after those who previously received cellular immunotherapy, with ECOG score >2, estimated survival time <12 weeks, loss of follow-up or incomplete clinical data were excluded. The patients who missed the follow-up were excluded again. There was no significant difference in gender, ages, date of hospitalization, clinical staging, surgery, radiotherapy, chemotherapy and OS as recorded between two groups (p>0.05).

*Statistical data analysis*

The statistical analysis was performed using SPSS 17.0 statistical package. Summary statistics were given for patient characteristics and treatment administration. Frequencies were reported by number and percentage. The data of phenotypes of lymphocyte cells in peripheral blood and harvested EAAL cells were expressed as means±standard deviation (x±s) and statistical comparison was made by self-paired t tests. Comparison of basic clinical characteristics between immunotherapy group and control group was made by Pearson chi-square test. OS was analyzed by means of Kaplan-Meier method and the differences in the distributions were compared by the log-rank test. Factors that might affect patients' OS were analyzed by means of COX multivariate regression method. Subgroup analysis was used to analyze the OS in different subgroups of patients who had received EAAL immunotherapy. p<0.05 was considered to be statistically significant.

## Results

*Characteristics of EAAL cell generation*

The proliferation of cells was observed 2 d after incubation under the inverted microscope. The cells grew in adherence to the flask wall at the beginning and then grew bigger and rounder and aggregated into cluster over time. When the cells matured, the adherent and aggregated cells were dislodged from the culture flask and then were suspended in the flask (Figure 1). It took (14.10±0.71) d to culture the cells into mature ones. The total number of cells was $(1.18\pm0.38)\times10^7$ before incubation and $(6.17\pm1.21)\times10^9$ after incubation, respectively. The amplification factor was (555.78±142.01) and the cell survival rate was (97.94±0.94)% (Table 1). Examination of endotoxin, bacterium, fungus, mycoplasma and adventitious viruses on harvest EAAL all showed negative (Figure 2).

*Phenotype alternations of lymphocytes before and after EAAL culture in vitro*

After culture and proliferation *in vitro*, the percentages of $CD3^+$, $CD3^+CD8^+$, $CD45RO^+$, $CD28^+$, $CD29^+$, $CD8^+CD28^+$ and $CD3^+CD16^+/CD56^+$ cells increased markedly (p<0.01), while those of $CD19^+$, $CD3^+CD4^+$, $CD45RA^+$, $CD4^+CD25^+$, $CD4^+CD29^+$ and CD3-$CD16^+/CD56^+$ (natural killer cells, NK) decreased obviously (p<0.01, Table 2).

*Basic clinical characteristics of patients*

Based on the records of cell therapy from May, 2008 to October, 2010, 23 cases that were pathologically diagnosed as SCLC and received EAAL treatment were selected. After eliminating the patients that conformed to the exclusion criteria, a total of 16 cases were included in EAAL treatment group. The patients in EAAL treatment group were divided into 2 subgroups, namely widespread group (10 cases) and limited group (6 cases). Ten cases in widespread stage and 6 cases in limited stage were randomly selected as the members of control group from 194 cases who had been admitted to the hospital in the same period. There were 14 males and 2 females in EAAL treatment group, 13 males and 3 females in control group. The patients in EAAL treatment group aged 38~78 years, with median age of 59.5, while the patients in control group aged 40~76 years, with median

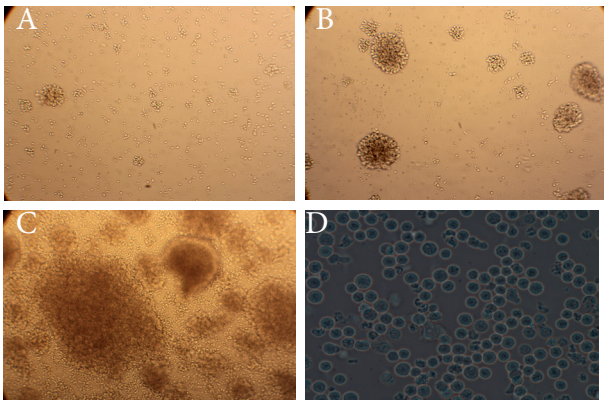


**Figure 1. Morphology of EAAL Cells at Different Intervals of Proliferation.** Peripheral blood lymphocytes of SCLC patients were harvested followed with in-vitro culture and stimulation. A: Day 2 (×10); B: Day 3 (×10); C: Day 7 (×10); D: Day 14 (×40)

**Table 1. EAAL Cell Properties**

| Property | Values |
|---|---|
| Time of proliferation (d) | 14.10±0.71 |
| Number of cells before proliferation (107/L) | 1.18±0.38 |
| Number of cells after proliferation (109/L) | 6.17±1.21 |
| Proliferation multiplicity | 555.78±142.01 |
| Survival rate of cells (%) | 97.94±0.94 |

**Table 2. Phenotype Alternations of Lymphocytes Before and after *in-vitro* Culture**

| Phenotype | Before (%) | After (%) | P |
|---|---|---|---|
| $CD3^+$ | 69.68±8.98 | 93.80±6.70 | 1.90263356599561E-57 |
| $CD3^+CD4^+$ | 38.78±9.56 | 13.84±10.01 | 1.52871890636846E-46 |
| $CD3^+CD8^+$ | 26.89±8.78 | 61.51±16.77 | 2.06152888632036E-47 |
| $CD3^+CD16^+/CD56+$ | 5.79±4.12 | 34.48±16.41 | 3.70181070248072E-43 |
| $CD3\text{-}CD16^+/CD56^+$ | 15.02±9.59 | 4.65±5.93 | 4.1069945361241E-18 |
| $CD19^+$ | 10.19±5.73 | 1.00±1.37 | 1.04799064480978E-38 |
| $CD29^+$ | 66.04±10.69 | 87.91±11.66 | 6.44354294431144E-33 |
| $CD25^+$ | 11.78±4.23 | 13.36±14.81 | 0.288 |
| CD4+CD29+ | 20.44±5.42 | 11.47±7.21 | 2.17504381591661E-20 |
| CD4+CD25+ | 8.36±3.14 | 0.73±1.33 | 5.94376450335784E-60 |
| CD45RA+ | 58.57±8.25 | 11.20±10.80 | 2.1252205267154E-92 |
| CD45RO+ | 49.82±10.28 | 91.87±12.27 | 5.37250131745158E-71 |
| CD28+ | 50.12±13.27 | 54.33±13.56 | 0.023 |
| CD8+CD28+ | 12.85±4.71 | 38.38±11.15 | 4.12623372517195E-56 |
| CD8+CD28- | 19.13±10.26 | 19.58±9.55 | 0.737 |

*Compared with in-vitro culture before, *p<0.05.

**Table 3. General Clinical Characteristics**

| Programs | EAAL treatment group (n) | Control group (n) | P |
|---|---|---|---|
| n | 16 | 16 | |
| Age | | | |
| <60 years | 8 | 8 | 1.0000 |
| ≥60 years | 8 | 8 | |
| Gender | | | |
| Male | 14 | 13 | 0.626 |
| Female | 2 | 3 | |
| Stage | | | |
| Limited | 6 | 6 | 1.0000 |
| Expand | 10 | 10 | |
| Brain metastasis | | | |
| Yes | 6 | 7 | 0.606 |
| No | 10 | 9 | |
| Surgery | | | |
| Yes | 1 | 2 | 0.544 |
| No | 15 | 14 | |
| Radiotherapy | | | |
| Yes | 13 | 14 | 0.626 |
| No | 3 | 2 | |
| Chemotherapy | | | |
| ≤6 cycles | 8 | 9 | 0.723 |
| >6 cycles | 8 | 7 | |

age of 60. In order to further make subgroup analysis, these patients were divided into 2 subgroups based on the age, namely group<60 years and group≥60 years, respectively. One case in EAAL treatment group and 2 cases in control group received the resection of primary tumor while 13 and 14 received radiotherapy and 6 and 7 developed cerebral metastasis upon assignment, respectively. Detailed general clinical characteristics of the enrolled patients are summarized in Table 3 and the statistical analysis showed that there was no significant difference between 2 groups (p>0.05).

*EAAL treatment and chemotherapeutic features*

EAAL treatment group received a total of 107 EAAL therapies, in which 2 were in the minimum, 23 in the maximum and 6 in the median. The total number of chemotherapy given to EAAL treatment group was 140 cycles, in which 4 in the minimum, 25 in the maximum and 6 in the median. In EAAL treatment group, 1 received adjuvant chemotherapy after surgery; 3 only received the

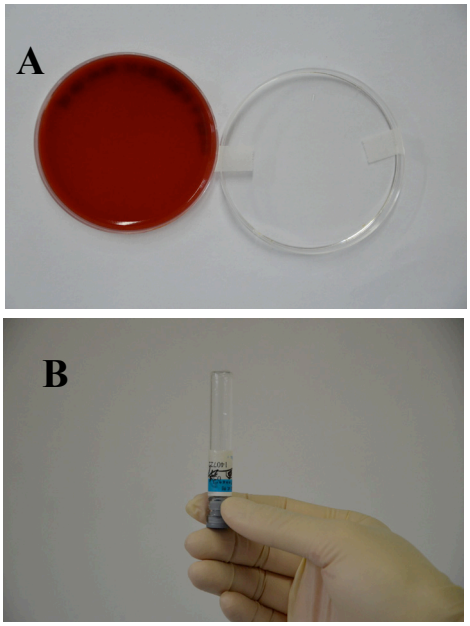


**Figure 2. Examination of Endotoxin and Bacterium.** A: Result of bacterium examination; B: Result of endotoxin examination

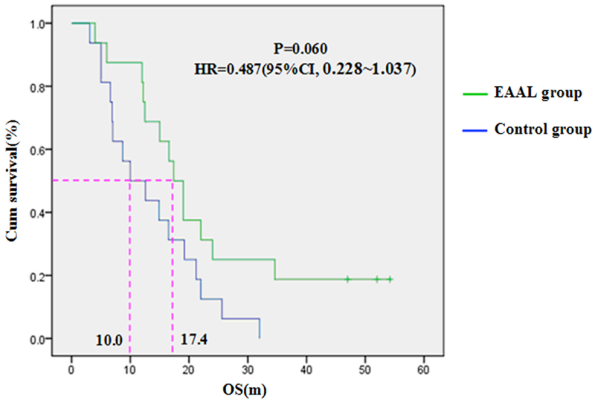


**Figure 3. Comparison of OS Between EAAL Treatment Group and Control Group**

**Table 4. Comparison of Survival Rates of SCLC Patients Between EAAL Treatment Group and Control Group (%) [95%CI]**

| Group | n | 1-year survival rate | 2-year survival rate | 3-year survival rate | 4-year survival rate | 5-year survival rate |
|---|---|---|---|---|---|---|
| SEAAL treatment group | 16 | 81.25[62.12, 100] | 25 [3.78, 46.22] | 18.75 [0, 37.88] | 18.75 [0, 37.88] | 18.75 [0, 37.88] |
| Control group | 16 | 43.75 [19.44, 68.06] | 6.25 [0, 18.11] | 0 | 0 | 0 |
| P | | >0.05 | >0.05 | | | |

**Table 5. COX Multivariate Regression Analysis of SCLC Patients**

| Factors | Wald | P | Hazard ratio (HR) | HR 95% CI |
|---|---|---|---|---|
| Gender | 0.026 | 0.872 | 0.907 | 0.278~2.957 |
| Age | 0.278 | 0.598 | 1.252 | 0.543~2.885 |
| Clinical stage | 1.565 | 0.211 | 1.711 | 0.738~3.969 |
| Surgery | 2.494 | 0.114 | 4.690 | 0.689~31.928 |
| Chemotherapy cycles | 5.213 | 0.022 | 2.801 | 1.157~6.783 |
| Radiotherapy | 3.508 | 0.061 | 3.797 | 0.940~15.337 |
| EAAL immunotherapy | 7.674 | 0.006 | 3.278 | 1.415~7.592 |

**Table 6. Subgroup Analysis**

| Subgroups | EAAL treatment group | Control group | RR | RR | P |
|---|---|---|---|---|---|
| Age | | | | | |
| <60 years | 19 | 10 | | 0.377 (0.113, 1.259) | 0.113 |
| ≥60 years | 15 | 8.7 | | 0.568 (0.205, 1.577) | 0.278 |
| Gender | | | | | |
| Male | 16.6 | 16.5 | | 0.933 (0.127, 6.876) | 0.946 |
| Female | 19 | 10 | | 0.428 (0.185, 0.992) | 0.048 |
| Clinical stage | | | | | |
| Limited disease | 17.4 | 14.9 | | 0.473 (0.141, 1.593) | 0.227 |
| Extensive disease | 15 | 6.9 | | 0.437 (0.161, 1.187) | 0.104 |
| Radiotherapy | | | | | |
| Yes | 19 | 12.6 | | 0.521 (0.23, 1.181) | 0.119 |
| No | 15 | 5 | | 0.342 (0.03, 3.851) | 0.385 |
| Chemotherapy cycles | | | | | |
| ≤6 cycles | 17.4 | 7 | | 0.14 (0.029, 0.684) | 0.015 |
| >6 cycles | 15 | 21.2 | | 0.852 (0.274, 2.483) | 0.732 |

**Table 7. Adverse Reactions [n (%)]**

| Event | Immunotherapy patients (n=16) | EAAL cellular transfer times (n=107) |
|---|---|---|
| Fever | 3 (18.75) | 7 (6.54) |
| Itching | 2 (12.50) | 3 (2.80) |
| Rash | 1 (6.25) | 2 (1.87) |
| Headache | 1 (6.25) | 2 (1.87) |
| Chill | 1 (6.25) | 1 (0.93) |
| Nausea | 1 (6.25) | 1 (0.93) |
| Tachycardia | 1 (6.25) | 1 (0.93) |
| Diarrhea | 1 (6.25) | 1 (0.93) |

first-line chemotherapy; 12 received the second-line and the multi-line chemotherapy; 1 received EAAL during adjuvant chemotherapy; 7 received EAAL during the first-line chemotherapy; 6 received EAAL during the second-line and the multi-line chemotherapy; and 2 received EAAL during the first-line and the second-line chemotherapy. The total number of chemotherapy in control group was 119 cycles, in which 4 were in the minimum, 14 in the maximum and 6 in the median. In control group, 1 case received adjuvant chemotherapy after surgery; 5 only received the first-line chemotherapy; and 10 received the second-line and the multi-line chemotherapy.

The chemotherapeutic regimens for study objects during the treatment included: CE (etoposide + carboplatin), EP (etoposide + cisplatin), IP (irinotecan + cisplatin), PP (paclitaxel + cisplatin/nedaplatin) and DP (docetaxel + cisplatin), CAV (cyclophosphamide +pharmorubicin + vincristine), NP (navelbine + cisplatin), pemetrexed alone, docetaxel alone, temozolomide capsule alone, etoposide capsule alone.

In order to further make subgroup analysis, these patients were assigned into two subgroups based on the cycles of chemotherapy, namely group≤6 cycles and group>6 cycles. The details of chemotherapeutic features of enrolled patients are listed in Table 3. Results showed that there was no significant difference between the two groups (p>0.05).

*Kaplan-Meier survival analysis*

At the endpoint of follow-up on December 31th, 2012, 13 patients (13/16, 81.25%) died in EAAL treatment group

and the median OS was 17.4 months; while in control group, all patients (16/16, 100.0%) died and the median OS was 10.0 months. All patients' deaths were associated with tumor progression. EAAL treatment group was longer than control group in OS time, but the difference was not statistically significant (p=0.06, HR=0.487, 95% CI 0.228~1.037, Figure 3). 1-, 2- and 3-year survival rates of EAAL treatment group were 81.25%, 25.0% and 18.75%,, evidently higher than the 43.75%, 6.25% and 0% in control group, respectively (Table 4).

*COX multivariate regression analysis*

COX multivariate regression analysis showed that the number of chemotherapy cycles and the application of EAAL immunotherapy were two independent risk factors for OS of SCLC patients (Table 5).

*Subgroup analysis*

Results of subgroup analysis are shown in Table 6. For the female subgroup, the median OS of EAAL treatment group was longer than that of control group (19.0 *vs*. 10.0 months, p=0.048). For the group≤6 cycles, the median OS in EAAL treatment group was also longer than that of control group (17.4 vs. 7.0 months, p=0.015). But for other subgroups, the median OS showed no significant difference between EAAL treatment group and control group (p>0.05).

*Security assessment of the EAAL treatment*

Eighteen adverse reactions developed in 107 EAAL transfer times, all of which were in grade 1 or 2 and were self-limiting (Table 7). No patient had pulmonary or renal symptoms or any sign of infection, hepatic functional deterioration or autoimmune disorder. There was no treatment-related death.

## Discussion

Tumor cells adopt diverse mechanisms to escape tumor-specific immunity in the neoplastic process. The pathological interactions between cancer cells and host immune cells not only create an immunosuppressive network in the tumor microenvironment but also are systemic (Boissonnas et al., 2013; Corthay et al., 2014). Transfusion of an adequate quantity of lymphocytes, which are capable of recognizing and lysing tumor cells, is the basis for successful adoptive cell therapy (Rajbhandary et al., 2013; Noguchi et al., 2014). Previous reports have suggested that T-cells from non-tumor-bearing hosts could boost the anti-tumor immunity to break the morbid equilibrium formed between tumor cells and the host (Vesely et al., 2013; Hosoi et al., 2014). Indeed, cell transfer therapy for cancer has been recognized as the fourth anticancer modality following the operation, chemotherapy and radiotherapy (Qian et al., 2014). However, the use of several immune cell types has been hampered by serious drawbacks including the poor efficacy and/or the complexity of cell propagation (Binsfeld et al., 2014; Kelderman et al., 2014; Weber et al., 2014). Interestingly, these shortcomings can be overcome through infusion of a large number of EAAL cells, as demonstrated in HCC (Takayama et al., 2000). An additional advantage of EAAL is that the use poses no risk of violating medical ethics since the effector cells are originated from the patient's PBMCs.

Herein, this study assessed a variety of molecular markers to further characterize the EAAL cell phenotypes. It was found that $CD3^+$ and $CD3^+CD8^+$ T lymphocytes represented more than 90% and 60% of the total EAAL cells, respectively, while the proportions of $CD3^+CD4^+$ and $CD3^-CD16^+/CD56^+$ NK cells were relatively lower. The proportions of $CD8^+CD28^+$ cytotoxic T lymphocytes (CTL) and CD3+CD16+/CD56+ T lymphocytes, which are essential effector cells and play an important role in anti-tumor immunity (Yu et al., 2013; Jakel et al., 2014; Wang et al., 2014), and which account for (38.38±11.15)% and (34.48±16.41)%, respectively, were also very high in EAAL cells. Therefore, the high content described above for these cell types in EAAL may result in increased anti-tumor immunity.

$CD45RA^+$ T cells are known as the "naive" T cells and $CD45RO^+$ T cells as the "memory" ones (Hara et al., 2007). High expression of $CD45RO^+$ in the EAAL cells in this study suggested that EAAL cells might be quickly activated when they were infused back to the patients and differentiated to cytotoxic cells if they encountered appropriate antigens, after which powerful anti-tumor response might emerged. The expression of CD29 is related to the migration and the invasion ability of tumor cells in tumor tissues. However, in peripheral blood lymphocytes, CD29 is usually expressed on the surface of the activated memory T cells (Leitner et al., 2010; Zhu et al., 2013; Song et al., 2014; Zhan et al., 2014). The percentage of $CD29^+$ cells accounts for (87.91±11.66)% in EAAL cells, which means the strong adhesion ability of EAAL cells. If tumor tissues could highly express the CD29 ligands, EAAL cells might easily penetrate into the tumor tissues and play the role of killing tumor cells.

The expression of regulatory T cell (Treg) specific transcription factors such as Foxp3 (Costantino et al., 2008; Wang et al., 2013) was not assessed in this study. However, the rather low percentage of CD4+CD25+ T cells [(0.73±1.33)%] implied that the proportion of Treg cells was extremely low in EAAL cells. These findings indicated that EAAL would not suppress the immunity in SCLC patients.

As a result, although not all lymphocytes are tumor-specific, the high expression of CD3, CD8, CD28, CD29, CD45RO, CD56 and CD16 in EAAL cells implies that a large number of EAAL cells do have the potential ability to exert or improve the anti-tumor effects.

In order to clarify the preliminary clinical effect of EAAL cells, this study adopted a case-control study to retrospectively analyze whether EAAL cells could prolong the OS time of SCLC patients. Comparison of basic clinical features revealed that there was no statistically significant difference between EAAL treatment group and control group (p>0.05), which suggested that the basic clinical features of EAAL treatment group and control group were similar and comparable.

Because EAAL immunotherapy was offered in different periods of the disease in different patients, this

study didn't analyzed the effect of EAAL immunotherapy on patients' disease-free survival (DFS) or progression-free survival (PFS) except the OS. The result of Kaplan-Meier survival analysis showed that the median OS of EAAL treatment group was longer than that of control group but the difference was not significant (median OS: 17.4 *vs*. 10.0 months, p=0.06, HR=0.487, 95% CI: 0.228~1.037). EAAL immunotherapy probably increased the 1- to 3-year survival rate of SCLC patients but the improvement also had no statistical significance, which might be associated with the reason that the research sample number was too small to get the positive result. If the sample size is increased, a positive result might be possible.

It was demonstrated by COX multivariate regression analysis that the number of chemotherapy cycles and EAAL immunotherapy were independent risk factors for OS in SCLC patients. The hazard ratio (HR) of the number of chemotherapy cycles was 2.801 (95%CI: 1.157~6.783) in this study suggested that patients in group>6 cycles might live longer than patients in group≤6 cycles. Similarly, the HR of the application of EAAL immunotherapy was 3.278 (95%CI: 1.415~7.592) suggested that the patients in EAAL treatment group might live longer than those in control group.

Results of subgroup analysis displayed that the OS of female and chemotherapy ≤6 cycles subgroups could be prolonged after EAAL cellular immunotherapy ($p<0.05$). The OS of other subgroups can also be improved after EAAL cellular immunotherapy, but the improvement was not significant ($p>0.05$).

As far as the safety concerned, the most common adverse reactions were fever (6.54%) and itching (2.80%). Other adverse reactions included rash, headache, chill, nausea, tachycardia and diarrhea, of which the incidence was no more than 2%. All the adverse reactions were in grade 1 or 2 and self-limiting, suggesting great safety of EAAL cellular immunotherapy and mild adverse reactions.

In conclusion, the in-vitro induction and proliferation method described in this study was easy and highly efficient, with good repeatability and biological safety. The data of this study suggested that EAAL cell immunotherapy might prolong the OS of SCLC patients. Meanwhile, great safety was obtained for EAAL cellular immunotherapy with only mild adverse reactions observed. However, this retrospective case-control study has a relatively small sample size, and prospective cohort clinical studies with larger sample sizes are still required for confirmation of these findings.

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