

Preparation of highly activated natural killer cells for advanced lung cancer therapy

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Background: Natural killer (NK) cells can be used as an adoptive immunotherapy to treat cancer patients.

Purpose: In this study, we evaluated the efficacy of highly activated NK (HANK) cell immunotherapy in patients with advanced lung cancer.

Patients and methods: Between March 2016 and September 2017, we enrolled 13 patients who met the enrollment criteria. Donor peripheral blood monocytes were isolated from patients and the NK cells were expanded. After 12 days of culture, the cells were collected and infused intravenously on days 13 to 15. The enrolled patients received at least one course including three times of infusions. The lymphocyte subsets, cytokine production, and the expression of carcinoembryonic antigen (CEA) and thymidine kinase 1 (TK1) were measured before treatment and after the last infusion.

Results: No side effects were observed. After a three-month follow-up, the percentage of patients who achieved stable disease and progressive disease was 84.6% and 15.4%. Moreover, the level of IFN- γ was significantly higher after treatment and the level of CEA decreased substantially. The overall immune function of the patients who received the NK cell therapy remained stable.

Conclusion: This is the first study to describe the efficacy of NK cell therapy of patients with advanced lung cancer. These clinical observations demonstrated that NK cell is safe and efficient for advanced lung cancer therapy.

Keywords: adoptive immunotherapy, highly activated natural killer cells, immune function, lung cancer

Introduction

Lung carcinoma is the most common type of cancer and the leading cause of cancer mortality in the People's Republic of China.¹ Lung cancer includes nonsmall cell lung cancer (NSCLC) and small cell lung cancer, of which NSCLC accounts for approximately 80% and is defined as the most dangerous and common malignant cancer.^{2,3} In addition, approximately 70% of patients with NSCLC are diagnosed at an advanced stage⁴ and the 5-year survival rate is only 16.8%.⁵ Surgery and chemotherapy are the standard therapies used to treat patients with NSCLC; however, they are insufficient to manage patients with advanced lung cancer due to a poor prognosis.^{6,7} Moreover, severe toxicity is exhibited following chemotherapy. Although biological therapy is an attractive alternative method for clinical treatment, variable therapeutic effects have been reported due to individual differences. Thus, the suppression of tumor cell proliferation in a complicated microenvironment remains a problem for researchers. Recently, progress in the field of adoptive

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immunotherapy has shown increased potential for the treatment of advanced lung cancer patients.^{8–10} However, a safe and efficient immunotherapy regimen is still required.

Natural killer (NK) cells are a critical component of the innate immune system and are characterized by their rapid response to and strong cytotoxicity against virus-infected or malignant cells without presensitization or restriction by major histocompatibility class I (MHC-I) molecules.^{11–13} NK cells identify their target cells through a set of activating and inhibitory receptors. Then, NK cells recognize self MHC-I molecules that are expressed on normal cells but downregulated by infected or transformed cells, which is termed the “missing-self” model.¹⁴ When target cells exhibit decreased self MHC-I molecule expression or when the activating signals (activating receptors on NK cells and their corresponding ligands on tumor cells) dominate over the balance of inhibitory signals (inhibitory receptors on NK cells and their ligands on tumor cells), NK cell cytotoxicity is triggered.¹⁵ Inhibitory signals mediated by killer cell immunoglobulin-like receptors and NK group 2A (NKG2A) on NK cells interact with MHC-I molecules that are expressed on target cells. By comparison, various activating receptors like NK group 2D (NKG2D) and the natural cytotoxicity receptors (NCRs), including NKp30, NKp44, and NKp46, on NK cells provide positive signals when activated.^{16,17}

NK cells are innate lymphocytes that are part of the first line of defense against tumor cells. In addition, NK cells can recognize and kill tumor cells without the requirement of prior antigen exposure. Recent studies have investigated the potential for NK cells to provide therapeutic benefit in patients with advanced lung cancer.^{18–21} Tumor cells can escape immune surveillance by downregulating the level of MHC molecule expression that releases NK cells from inhibition and initiates antitumor activities.²² With increased knowledge into NK cell function, adoptive NK cell therapy has been applied as a clinical treatment for advanced cancer patients, including lung cancer.^{23–27}

To enhance the immune function of lung cancer patients, we isolated NK cells from patients themselves as adoptive immunotherapy. We developed the method to expand the NK cell number by a100-fold in 2 weeks with purity level $\geq 80\%$, and the expression level of the activating receptor increased almost 200-fold.²⁸ We also reported a case in which an advanced ovarian cancer patient received highly activated NK (HANK) cells

cultured and proliferated ex vivo by this approach and had a good response.²⁹ Therefore, we adopted HANK cells to treat lung cancer patients in the clinical trial.

In this study, we assessed the clinical effect of HANK cell therapy in patients with advanced lung cancer, as a potential novel therapeutic regimen.

Materials and methods

Ethics

This clinical trial was approved by the Guangzhou Fuda Cancer Hospital ethics committee. In accordance with the Declaration of Helsinki, written informed consent was obtained from each participant.

Patient eligibility

Patients were enrolled in the present study based on the following criteria: 1) life expectancy >3 months; 2) age >18 years; 3) Karnofsky performance status >60 ; 4) pathological or radiographic confirmation of stage III–IV lung cancer; 5) no serious abnormalities in liver, lung, and kidney function; 6) no high blood pressure, acute or chronic infection, or severe heart disease; 7) no HIV, HTLV-1, syphilis, tuberculosis, or parasitic infections; and 8) the absence of level 3 hypertension, severe coronary disease, myelosuppression, and autoimmune diseases.

Preparation of HANK cells

HANK cells were prepared under good manufacturing practice conditions using clinical-grade reagents. The human NK cell in vitro culture kit (HAHK Bioengineering Co. Ltd, Shenzhen, People's Republic of China) was used to costimulate expansion and activation of NK cells in the peripheral blood mononuclear cells (PBMCs) according to the manufacturer's instructions. Briefly, collect approximately 50 mL of peripheral blood from the patient. Transfer the blood to a 50-mL conical tube and centrifuge at $600\times g$ for 15 min. Collect the plasma in the supernatant in a 50-mL conical tube. Add an equal volume of saline to the blood cells in the bottom, and resuspend the cells for lymphocyte separation. Transfer the plasma tube to a $56\text{ }^{\circ}\text{C}$ water bath for 30 min, then stand on the bench top until the tube temperature is below $37\text{ }^{\circ}\text{C}$, centrifuge at $400\times g$ for 10 min, and transfer the supernatant into a new 50-mL conical tube and store at $4\text{ }^{\circ}\text{C}$ for further applications. The plasma tube stored at $4\text{ }^{\circ}\text{C}$ for more than 12 h should be centrifuged again and only the supernatant used. Transfer 20 mL human lymphocyte separation solution (Haoyang Biological Manufacture Co., Ltd,

Tianjin, People's Republic of China) into two 50-mL conical tubes, respectively. Carefully lay equal volumes of the blood cell suspension onto the lymphocyte separation solution in two 50-mL conical tubes, centrifuge at $600\times g$ for 15 min, transfer the lymphocytes into the middle layer into a 50-mL conical tube, and wash twice with saline. The total cell number is counted.

NK cell culture media consist of 1 L of X-Vivo15 serum-free medium (Lonza, Walkersville, MD, USA) and one tube of HK-002 (5% for initial 200 mL of medium, then reduce to 1–2%). A tube of HK-001 contains membrane chimeric active cellular factors. One tube of HK-001 is good for 4×10^7 lymphocyte initial culture. Calculate the number of HK-001 tubes needed according to the lymphocyte numbers. Take HK-001 out of the liquid nitrogen or $-80\text{ }^{\circ}\text{C}$ freezer, and immediately put into a $37\text{ }^{\circ}\text{C}$ water bath to recover. Then centrifuge at $350\times g$ for 5 min, and remove the supernatant. Wash the precipitate twice with saline. Resuspend the precipitate with about 3 mL NK cell culture medium.

The NK cells were cultured as follows. On day 1, mix 4×10^7 lymphocytes, 50 mL NK cell culture medium, and one tube of recovered HK-001 in a T175 culture flask (Corning Incorporated, Corning, NY, USA) and incubate at $37\text{ }^{\circ}\text{C}$ with 5% CO_2 . On day 3, add 30 mL NK cell culture medium to the T175 flask. On day 5, add 60 mL NK cell culture medium to the T175 flask and adjust the cell concentration to $1\times 10^6/\text{mL}$. On day 6, add 60 mL NK cell culture medium to the T175 flask and, adjust the cell concentration to $1\times 10^6/\text{mL}$. On day 7, add 50 mL NK cell culture medium (1–2% plasma concentration) to the T175 flask. Count the cell number. If the total cell number is $>6\times 10^7$ cells, add a tube of recovered HK-

001; or if the total cell number is $3\sim 6\times 10^7$ cells, add a tube of recovered HK-001 1 day later. Perform the first sterility test. On day 8, transfer the total culture from the T175 flask to the 2-L cell culture bag (Haoyang Biological Manufacture Co., Ltd). Add 200 mL NK cell culture medium to the cell culture bag. On days 9, 10, and 11, add 150 mL NK cell culture medium to the cell culture bag each day. On day 12, add 350 mL NK cell culture medium to the cell culture bag. Perform the quality control tests including cell viability, NK cell purity, and endotoxin. On day 13, harvest the HANK cells. Count the total cell number (should be around 1×10^{10} cells). Collect the cultures into 450-mL conical centrifuge tubes. Precipitate cells and wash the cells once with saline. Adjust the cell concentration to $2\times 10^7/\text{mL}$ with cell infusion solution (400 mL saline with 1% human serum albumin and 6 mL HK-003). A sample of $3\sim 5\times 10^9$ HANK cells was harvested into blood transfusion bags each day for infusion at a concentration of $2\times 10^7/\text{mL}$. The release tests were performed on each bag of cells. The process of HANK cell preparation is shown in Figure 1.

NK cells were stained with anti-CD3-APC-H7, anti-CD16-Alexa Fluor700, anti-CD56-PE, anti-NKG2D-PerCP-Cy5.5, anti-NKG2A-PE-Cy7, anti-NKp30-APC, anti-NKp44-PE, and anti-NKp46-BV786 (BD Biosciences, San Jose, CA, USA). Then, the cells were washed with fluorescent activated cell-sorting buffer (0.2% BSA) (Hyclone), fixed with 1% paraformaldehyde (Sigma-Aldrich Co., St Louis, MO, USA), and analyzed on the FACSCanto™ II (BD, Franklin Lakes, NJ, USA). Data analysis was performed using FlowJo software (Treestar). Cells were gated on $\text{CD56}^+\text{CD3}^-$ events (NK cells) and then analyzed for the expression of inhibitory and

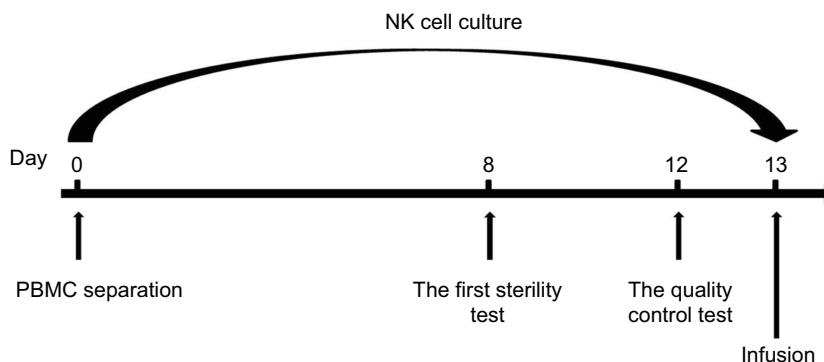


Figure 1 NK expansion process. PBMCs were obtained from peripheral blood (approximately 50 mL) of each cancer patient. The plasma was prepared, and the lymphocytes were separated using human lymphocyte separation solution. The lymphocytes were resuspended in NK cell culture medium and cultured. For the first week, the cells were cultured in a T175 culture flask. From day 8, the cells were cultured in a 2-L cell culture bag. The first sterility test was performed on day 8, while the quality control (cell viability $\geq 80\%$, NK cell purity $\geq 80\%$, endotoxin ≤ 1 EU/mL, and bacteria, fungi, and mycoplasma culture-negative) and sterility tests were performed on day 12. On day 13, approximately $3\sim 5\times 10^9$ HANK cells were harvested into a blood transfusion bags each day for infusion at a concentration of approximately $2\times 10^7/\text{mL}$.

Abbreviations: NK, natural killer; PBMC, peripheral blood mononuclear cell; HANK, highly activated natural killer.

activating surface receptors. The assessment was performed in freshly isolated peripheral blood mononuclear cells (PBMCs) on day 0 as well as expanded and activated NK cells on day 12.

Therapeutic procedure

Enrolled patients stopped pretreatment before HANK cell therapy. All enrolled patients received at least one course of treatment (each course included three infusions, the length of time for each infusion was within 30 min). No more than three courses of treatment were received monthly. Each course with three infusions was delivered consecutively over 3 days.

Evaluation of safety and clinical efficacy

Adverse effect observation

The most common adverse reactions recorded include local (eg, pain and retroperitoneal erythema) and systemic (eg, chills, fatigue, and fever) reactions. Blood toxicity was measured by the white blood cell count (reference: $3.5\text{--}9.5 \times 10^9$ cells/L) and the hemoglobin level (reference: 130–175 g/L). Hepatic toxicity was measured by aspartate transaminase (reference: 15–40 U/L) and alanine aminotransferase ALT (reference: 9–50 U/L). Renal toxicity was measured by creatinine (reference: 57–97 $\mu\text{mol/L}$) level. All of these indexes were measured 14 days before and after HANK cell infusion as a detection index to evaluate adverse effects.

Detection of immune function

Lymphocyte subsets were detected by flow cytometry (FACSCanto™ II; BD). Peripheral blood (2 mL) was drawn to assess the level of immune function before and after the HANK cell infusion. The number of CD3⁺CD8⁺ (reference range: 125–1312 cells/ μL), CD3⁺CD4⁺ (reference range: 441–2156 cells/ μL), CD3⁻CD16⁺CD56⁺ (reference range: 95–640 cells/ μL), total CD3⁺ (reference range: 603–2990 cells/ μL), and CD3⁻CD19⁺ (reference range: 107–698 cells/ μL) cells was detected by the BD Multitest 6-color TBNK Reagent (BD Biosciences). The BD Cytometric Bead Array Human Th1/Th2 Cytokine Kit II (No. 551,809; BD) was used to detect the expression of IL-2 (reference range: 8–12.5 pg/mL), IL-4 (reference range: 3.5–6 pg/mL), IL-6 (reference range: 2.7–8.5 pg/mL), IL-10 (reference range: 1.8–4 pg/mL), tumor necrosis factor- β (reference range: 1.7–2.5 pg/mL), and interferon- γ (reference range: 1.5–4 pg/mL). These factors are inhibited by tumor proliferation, and the level of these

cytokines is indicative of the potency of the antitumor response elicited by the immune system.³⁰

Tumor biomarkers

The level of carcinoembryonic antigen (CEA) and thymidine kinase 1 (TK1) was measured. TK1 is associated with DNA synthesis related to cellular proliferation and is used to monitor the effect of tumor therapy, prognosis, and follow-up. In addition to TK1, CEA is a tumor-associated antigen that is an important marker for lung cancer. Peripheral blood (5–6 mL) was collected for the detection of TK1 and CEA. The TK1 analytical reagent kit and CIS-2 series chemiluminescence digital imaging analyzer (Sino-Swed Tong Kang Bio-Tech Co. Ltd., Shenzhen, People's Republic of China) were used to detect the TK1 level (normal range: 0–2.0 pM) before and after the HANK cell immunotherapy. The expression level of CEA was detected by a chemiluminescent immunoassay.

Computed tomography imaging scan

Changes in computed tomography tumor imaging were monitored to evaluate the curative effect of the treatment according to the evaluation standards published by the World Health Organization.³¹ According to the degree of change in the largest transverse diameter, the therapeutic effect is categorized as: 1) complete response (CR), in which there is a disappearance of the arterial enhancement imaging of all target lesions; 2) partial response (PR), in which there is a total reduction in the diameter of the target lesions >30%; 3) stable disease, in which tumor regression fails to reach PR or tumor progression fails to reach progressive disease; and 4) progressive disease, a total progression of the tumor diameter >20%. To accurately observe the therapeutic effects of HANK cell therapy, the total area of all tumors before and after treatment was compared. Any curative effect must be maintained for more than 4 weeks, with CR+PR representing the effective rate (RR).

Statistical analysis

SPSS version 13.0 (SPSS Inc., Chicago, IL, USA) was used for the statistical analyses, and the results were expressed as the mean and SD values. GraphPad Prism 5 (GraphPad Software Inc., La Jolla, CA, USA) was used to plot graphs. The two groups (immune function and tumor markers) were compared using Wilcoxon signed-rank tests. All statistical tests were two-sided, and differences were considered significant at $p < 0.05$.

Results

Patient characteristics

From March 2016 to September 2017, a total of 13 patients (eight males, five females) with a median age of 57.3 years (range: 37–84 years) and a diagnosis of adenocarcinoma (n=12) or squamous cell carcinoma (n=1) were enrolled in the study. Among these patients, stage IV, III, and II was detected in 10, 2, and 1 patient, respectively (Table 1).

Treatment safety

Before HANK cell therapy, all other therapies were stopped. The detailed pretreatment data are shown in Table 2. During all infusions, the patients did not report a cold chill, fever, or any other discomfort. There was no case of queasiness or vomiting. After 14 days of HANK cell infusion, the white blood cell count and the aspartate transaminase, alanine aminotransferase, hemoglobin, and creatinine levels were not significantly different from those before treatment ($p>0.05$) (Table 3). It was demonstrated that blood function, liver function, and renal function remained at a normal level.

NK cell purity before and after in vitro expansion and activation

Prior to NK cell expansion, the median percentage of the CD3⁺CD56⁺ population in PBMC was 13.4% (range: 9.4–16.8%). Following expansion, the proportion of viable cells exceeded 92% without any bacterial, fungal, or mycoplasma contamination (endotoxin level <1 EU). The median

proportion of CD3⁺CD56⁺ cells was 88.1% (range: 81.3–94.9%). The representative results are presented in Figure 2.

We also examined the expression rates of various activating and inhibitory receptors on the NK cell surface. The results show that there is a significant increase in the expression of activating receptors after culture compared to those on PBMC before expansion (Figure 3). For instance, NKG2D/CD56⁺ NK cells increase from 24% in PBMCs to 95% after 12 days of NK cell culture. Similarly, NKp30/CD56⁺ NK cells, NKp44/CD56⁺ NK cells, and NKp46/CD56⁺ NK cells were increased from 22%, 0.5%, and 62% to 65%, 66%, and 78%, respectively. Moreover, expansion and activation of NK cells had no influence on NKG2A/CD56⁺ NK cells with a stable change from 14% on day 0 to 12% on day 12.

Immune function changes in patients with advanced lung cancer following HANK cell therapy

The level of interferon- γ was significantly higher after treatment ($p<0.05$). In addition, the lymphocyte subset number remained stable (Table 4). No change was observed regarding the level of cytokines, including IL-2, IL-4, IL6, IL-10, and tumor necrosis factor- β ($p>0.05$) (Table 5).

Changes in tumor biomarker expression

The level of CEA significantly decreased following HANK cell adoptive immunotherapy ($p<0.05$). The mean TK1 value before HANK cell immunotherapy was 2.08 ± 4.35 pmol/L and increased to 2.7 ± 5.78 pmol/L after the

Table 1 Patient characteristics

No.	Age (years)	Gender	Histology stage	Smoking status	Metastasis
1	84	M	A IV	N	Bone
2	37	M	A IV	N	Bone
3	57	M	A IIa	N	No
4	48	M	A IV	N	Liver
5	55	F	A IV	N	Bone
6	57	F	A IV	N	Lung
7	54	F	SCC IV	N	Bone
8	82	M	A IIIb	N	No
9	45	M	A III	N	Bone, liver
10	62	F	A IV	N	Bone
11	60	F	A IV	N	Lung
12	37	M	A IV	10 years, 5 cigarettes a day	Bone, lung
13	67	M	A IV	N	Bone, lung

Abbreviations: M, male; F, female; A, adenocarcinoma; SCC, squamous cell carcinoma; N, no smoking history.

Table 2 Pretreatment data

No.	Size of primary lesion (cm)	Therapeutic regimen	Result
1	5.2×5.0	Mar and Apr 2016: cryoablation+iodine seed implantation	SD
2	5.8×7.7	Oct 2015: radiotherapy	SD
3	1.8×1.6	Oct 2015–Apr 2016: chemotherapy May and Jun 2016: cryoablation+iodine seed implantation	PD
4	3.9×2.8	Oct 2015–Oct 2016: targeted therapy Jun 2017: cryoablation+iodine seed implantation	PD
5	1.5×1.6	Dec 2014–Mar 2016: chemotherapy May 2016: cryoablation+iodine seed implantation	SD
6	2.8×1.8	Apr and Aug 2016: surgery+chemotherapy May 2015: cryoablation Jun and Sep 2015: chemotherapy Jan 2016: cryoablation	SD
7	2.5×1.4	Jan 2016: chemotherapy	SD
8	4.4×2.2	Jan 2016: cryoablation	SD
9	2.0×1.7	Jun 2015: surgery	SD
10	1.4×2.7	Nov 2011 so far: chemotherapy	SD
11	1.7×1.3	May 2015: surgery Jan 2016: chemotherapy	SD
12	2.7×2.9	Nov 2015: targeted therapy	SD
13	8.2×5.6	Jan and Mar 2010 so far: radiotherapy+chemotherapy Aug 2016: cryoablation+chemotherapy	SD

Abbreviations: SD, stable disease; PD, progressive disease.

Table 3 Detection index of adverse effects

	WBC ($10^9/L$)	Hb (g/L)	AST (U/L)	ALT (U/L)	Cr ($\mu\text{mol/L}$)
Before treatment	6.95±1.74	124.15±18.7	26.77±23.49	28.54±20.1	56.38±12.95
After treatment	6.37±2.53	118±22.13	18.46±6.66	21.62±4.44	49.54±12.2
p-value	0.307	0.272	0.073	0.556	0.196

Note: Values shown are the mean±SD. * $p < 0.05$.

Abbreviations: WBC, white blood cell count; Hb, hemoglobin; AST, aspartate transaminase; ALT, alanine aminotransferase; Cr, creatinine.

last transfusion. Thus, there is no substantial change in the level of TK1 (Table 6).

Clinical outcomes

A total of 13 patients received different courses of autologous HANK cell infusion. After 3 months, 11 patients (84.6%) experienced stable disease and 2 patients (15.4%) experienced progressive disease according to the RECIST guidelines (Table 7).

Discussion

Lung cancer is reported to be a major cause of mortality and is associated with the highest incidence among all malignancies worldwide.³² Furthermore, the majority of patients are diagnosed with advanced or metastatic disease.³³ Although chemotherapy and radiotherapy can

be effective to some extent, the curative and beneficial effects of these treatments are severely limited.^{34,35}

Adoptive immunotherapy has long been recognized as a promising novel approach for the treatment of solid tumors.³⁶ As a part of the innate immune system, NK cells are highly related to the tumor microenvironment and mediate cytotoxic activity against tumor cells.³⁷ Thus, the clinical application of NK cell adoptive immunotherapy as a potential treatment for patients with solid tumors has recently gained attention.^{38–40}

A previous study has shown that NK cells can recognize and lyse cells lacking MHC-I molecules through their activating receptors like NKG2D, NKp46, NKp44, and NKp30.^{16,17} Hence, tumor cells are highly susceptible to NK cells due to their lack of MHC-I molecule expression.¹⁴ In addition, the balance between activating and inhibitory

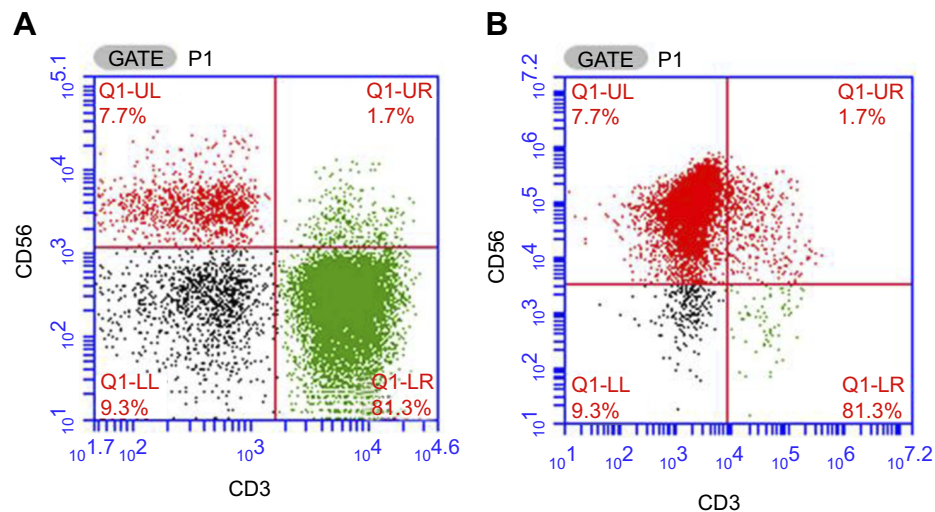


Figure 2 The CD3⁻CD56⁺ NK cell proportion before and after in vitro expansion. (A) The proportion of NK cells before the expansion. (B) The proportion of NK cells after expansion on day 12.

Abbreviation: NK, natural killer.

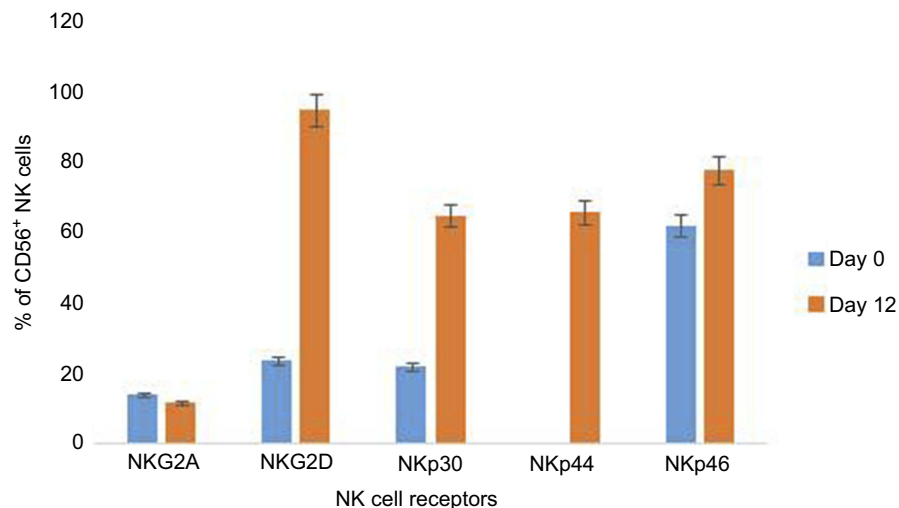


Figure 3 Effects of ex vivo expansion on expression of NK cells. NK cells were stained with conjugated antibodies like PE or APC, etc., against activating receptors such as NKG2D, NKp30, NKp44, and NKp46 or an inhibitory receptor such as NKG2A. Flow cytometry was performed after incubation. The presented data show the results from one donor. The error bars represent SDs, according equal weight to all three assays.

Abbreviations: NK, natural killer; NKG2, NK group 2.

receptor signaling on NK cells is crucial to NK cell functionality, including cytokine production, cytotoxicity mediated by NK cells such as perforin and granzyme, and antibody-dependent cell-mediated cytotoxicity.^{41–46} Depending on the balance between the activating and inhibitory signals engaged by ligands expressed on tumor cells, NK cells are triggered to kill or ignore target cells. Data from recent studies indicate that in response to NK cell therapy, the expression of NK cell activating receptors like NKG2D increased, whereas the expression of inhibitory receptors did not, suggesting that NK cell therapy can activate NK

cells in advanced cancer patients.^{23,47} However, the clinical application of NK cells has been limited by the low frequency of NK cells in peripheral blood (5–20%). Moreover, the expansion of NK cells to abundant numbers in vitro also remains difficult.

A recent study reported that expansion of NK cells can be achieved by inducing proliferation in response to the presence of feeder cells, such as irradiated PBMC, Epstein–Barr virus-transformed lymphoblastoid cell lines, and gene-modified K562 cells, which act as artificial antigen-presenting cells.^{24,48–50} In particular, feeder

Table 4 The number of lymphocytes isolated from lung cancer patients before and after highly activated natural killer cell immunotherapy

Lymphocytes	Before treatment (cells/ μ L)	After treatment (cells/ μ L)	p-value
Total T cells	754.5 \pm 391.858	763.1 \pm 499.418	0.807
CD4 ⁺ T cells	399.5 \pm 264.015	424.7 \pm 321.786	0.972
CD8 ⁺ T cells	343.1 \pm 256.85	379.7 \pm 311.261	0.552
B cells	108.7 \pm 91.846	115 \pm 92.022	0.753
Natural killer cells	150.2 \pm 91.846	198.9 \pm 149.81	0.064

Table 5 The level of cytokines expressed in peripheral blood before and after highly activated natural killer cell immunotherapy

Cytokine	Before treatment	After treatment	p-value
IL-2	14.02 \pm 2.915	14.48 \pm 2.51	0.345
IL-4	10.36 \pm 2.485	9.71 \pm 0.724	0.463
IL-6	22.98 \pm 9.26	26.13 \pm 18.88	0.972
IL-10	8.79 \pm 1.349	11.83 \pm 6.14	0.101
TNF- β	5.24 \pm 0.5	5.28 \pm 0.64	0.65
IFN- γ	6.55 \pm 0.64	7.79 \pm 2.25	0.039 [△]

Note: Values shown are the mean \pm SD. [△]p<0.05

Abbreviations: TNF, tumor necrosis factor; IFN, interferon.

Table 6 The level of CEA and TKI prior to and following highly activated natural killer cell adoptive immunotherapy

Expression	Before treatment	After treatment	p-value
CEA (ng/ml)	65.68 \pm 99.27	31.63 \pm 62.2	0.009 [△]
TKI (pmol/L)	2.08 \pm 4.35	2.7 \pm 5.78	0.382

Note: Values shown are the mean \pm SD. [△]p<0.05.

Abbreviations: CEA, carcinoembryonic antigen; TKI, thymidine kinase I.

cells based on genetically modified K562 cells that contain chimeric active cellular factors on K562 cell membranes produce a high quantity of NK cells.^{51,52} The human NK cell in vitro culture kit used in this study contains membrane chimeric cellular factors. However, a concern is that the in vitro expanded NK cells may reduce their cytotoxicity and persistence in vivo within several weeks. Ready to take fast action but a short persistence time are the advantage and disadvantage of adoptive NK cell therapy. Thus, repeated infusions are needed.

A series of studies have demonstrated that NK cell therapy is a promising therapeutic approach to advanced

Table 7 Clinical outcome of highly activated natural killer cell treatment after 3 months

No.	Time of transfusions	Clinical outcome
1	Apr 2016: 1 course	SD
2	Jun 2016: 1 course	SD
	Aug 2016: 1 course	
3	Oct 2016: 1 course	PD
	Jul 2017: 1 course	
4	Aug 2017: 2 courses	PD
	Sep 2017: 1 course	
5	Oct 2016: 1 course	SD
	Sep 2016: 1 course	
6	Oct 2016: 3 courses	SD
	Jul 2016: 1 course	
	Aug 2016: 2 courses	
	Sep 2016: 2 courses	
7	Oct 2016: 2 courses	SD
	Nov 2016: 1 course	
	Dec 2016: 2 courses	
	Jan 2017: 2 courses	
8	Feb 2017: 1 course	SD
	Jul 2016: 1 course	
	Aug 2016: 2 courses	
	Sep 2016: 2 courses	
9	Oct 2016: 2 courses	SD
	Mar 2016: 1 course	
10	Jul 2016: 1 course	SD
	Sep 2016: 2 courses	
	Oct 2016: 2 courses	
11	Sep 2016: 1 course	SD
	Oct 2016: 1 course	
12	Sep 2016: 1 course	SD
	Oct 2016: 2 courses	
13	Sep 2016: 1 course	SD
	Oct 2016: 1 course	
	Nov 2016: 1 course	
	Dec 2016: 1 course	
	Jan 2017: 1 course	
	Feb 2017: 1 course	
	Apr 2017: 1 course	

Abbreviations: SD, stable disease; PD, progressive disease.

lung cancer. Tonn et al⁵³ observed that some encouraging responses in patients with advanced lung cancer after two infusions of natural killer cell line NK-92. Yang et al²⁶ found that treatment of NK cell-rich lymphocytes combined with docetaxel in patients with advanced NSCLC was feasible without further toxicity or complication. Other regimens such as targeted therapy and percutaneous cryoablation combined with NK cell immunotherapy in advanced NSCLC patients also achieved satisfactory results.^{54,55} In addition, some

clinical studies have been performed well in various tumors combined with chemotherapy or antibody.^{27,56} It is not only safe and feasible, but also a better immune response is achieved when NK cell therapy is applied in cancer patients.^{57,58}

In summary, we have developed a practical, simple, safe, and economical NK cell expansion and activation procedure, which result in NK cells are with high quantity, high purity and high activity characterizations. Application of these NK cells for advanced lung cancer immunotherapy was safe and efficient, most of them remained in a stable condition. Although this NK cell therapy shows promise as a potential cancer treatment, further studies are needed to obtain additional improved curative effect.

Disclosure

The authors report no conflicts of interest in this work.

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