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Therapeutic outcomes of combining cryotherapy, chemotherapy and DC-CIK immunotherapy in the treatment of metastatic non-small cell lung cancer ☆

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ABSTRACT

Currently there are no effective therapies for the treatment of metastatic non-small cell lung cancer (NSCLC). Here, we conducted a retrospective study of 161 patients to evaluate the therapeutic effects of combining cryosurgery, chemotherapy and dendritic cell-activated cytokine-induced killer cells (DC-CIK) immunotherapy. The overall survival (OS) after diagnosis of metastatic NSCLC to patient death was assessed during a 5-years follow-up period. OS of patients who received comprehensive cryotherapy was (median OS, 20 months; $n = 86$) significantly longer than that of patients who did not received cryotherapy (median OS, 10 months; $n = 75$; $P < 0.0001$). Five treatment combinations were selected: chemotherapy ($n = 44$); chemo-immunotherapy ($n = 31$); cryo-chemotherapy ($n = 32$); cryo-immunotherapy ($n = 21$); and cryo-chemo-immunotherapy ($n = 33$). A combination of cryotherapy with either chemotherapy or immunotherapy lead to significantly longer OS (18 months and 17 months, respectively) compared to chemotherapy and chemo-immunotherapy (8.5 months and 12 months, respectively; $P < 0.001$); however, the median OS of patients who underwent cryo-chemo-immunotherapy was significantly longer (27 months) compared to the other treatment programs ($P < 0.001$). In conclusion, a combination of cryotherapy, chemotherapy and DC-CIK immunotherapy proved the best treatment option for metastatic NSCLC in this group of patients.

Introduction

Lung cancer is one of the leading causes of cancer deaths worldwide, with a 5-years survival rate of only 15% [19]. Approximately 80% of lung cancers are non-small-cell lung carcinoma (NSCLC) [17] with over 50% of patients with NSCLC exhibiting advanced local invasion and distant metastasis. Some patients with NSCLC are not suitable for surgery, but are suitable for other treatments such as chemotherapy, cryotherapy or immunotherapy [6]. Currently, chemotherapy is the standard treatment for advanced stage and metastatic NSCLC [43]; however, chemotherapy is associated with a decline in sensitivity over time and often has a toxicity profile that reduces quality of life without significantly improving prognosis [41]. Cryotherapy (also known as cryoablation), a minimally invasive ablation procedure, has been used as a local therapy for malignancies, including prostate cancer, hepatocellular carcinoma, pancreatic cancer and lung cancer [5,20,25,34]. A potential advantage of *in situ* freezing of malignant tumors is the cryo-immunologic response [24,39], which is an antitumor immune response that triggers the natural absorption of malignant tissue [38]. Immunotherapy works on the premise that the immune system can distinguish cancerous cells from normal cells; this technique benefits from a low toxicity and high specificity and is emerging as a new approach for long-lasting disease control in various types of cancer, including NSCLC [15]. Several studies have shown that autologous dendritic cells (DCs) can activate cytokine-induced killer (CIK) cells to enhance antitumor effects in patients with NSCLC [44]. In this study, the therapeutic outcomes, including overall survival (OS), of combined chemotherapy, cryotherapy and DC-CIK immunotherapy were retrospectively investigated in patients with metastatic NSCLC over a 5-years follow-up period.

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Materials and methods

Ethical approval

The study protocol was approved by the regional ethics committee at Fuda Hospital and Fuda Cancer Hospital, Guangzhou, China. Written informed consent was obtained from each participant prior to the study, in accordance with the Declaration of Helsinki.

Patient selection

This retrospective study enrolled patients treated for metastatic NSCLC in Fuda Cancer Hospitals between October 2007 and August 2012. Patients had received no surgical treatment prior to hospitalization and were diagnosed with metastatic disease after undergoing comprehensive evaluation by a multidisciplinary team, including a radiologist, a lung surgeon and an oncologist. The diagnoses were primarily based on computed tomography (CT) imaging and histological examination of fine-needle biopsies. All patients received their final treatments within the 5-years follow-up period.

Surgery was considered unsuitable for all of the enrolled patients for the following reasons: multiple metastases diffuse lung tumors, unresectable lung tumors, severe complications (e.g., hypertension, hydrothorax or ascites), advanced age, or refusal to undergo surgery. The inclusion criteria were as follows: Karnofsky Performance Status (KPS) score $\geqslant 70$, platelet count $\geqslant 80 \times 10^9$/L, white blood cell count $\geqslant 3 \times 10^9$/L, neutrophil count $\geqslant 2 \times 10^9$/L, hemoglobin level $\geqslant 90$ g/L and Prothrombin Time and International Normalized Ratio $\geqslant 1.5$. The diameter of the largest primary or metastatic tumor was <8 cm (as measured by preoperative CT); and the sites were isolated, not diffuse, and not obviously invading large blood vessels or nerves, with the central lung tumor not infiltrating or blocking the main bronchi. The preferred treatment for patients with primary or metastatic tumors with a diameter $\geqslant 8$ cm, or obvious invasion of the large blood vessels or nerves is vascular interventional embolism; as such, these patients were not enrolled in this study. All patients had to show normal baseline liver function and ascites <1 L, normal renal function, an absence of brain metastasis and have no history of level 3 hypertension, severe coronary disease, myelosuppression, respiratory disease, acute and chronic infection, or pulmonary functional compensation. In addition, a complete clinical record had to be available for all enrolled patients. A total of 161 patients met these inclusion criteria and were recruited to the study.

Cryotherapy

Percutaneous cryoablation was performed on 86 patients, followed by additional chemotherapy, immunotherapy, or both. Cryoablation was performed under general anesthesia (termed comprehensive cryoablation, as previously described [29,30]) and obvious intra- and extrapulmonary masses were completely, or mostly, treated. The procedures were performed under guidance from double-row helical CT (Somatom Emotion Duo; Siemens, Germany) or color ultrasonography (ALOKA SSD-5500SA; Aloka, Japan). Each surgery consisted of 2–3 freeze–thaw cycles using an argon gas-based cryosurgical unit (Endocare, Irvine, CA, USA). Depending on the location of the lung tumor, the cryoprobes (Cryo-42; Endocare) were inserted percutaneously via the scapular line, the posterior axillary line or the rib margin; for metastatic lesions, the cryoprobes were inserted percutaneously to the center of tumor; for tumors with a major diameter >3 cm, cryoprobes $\geqslant 2$ were used. Care was taken not to puncture the large nerves or blood vessels. After the cryosurgery was completed, fibrinogen and thrombin (1 ml of each) were simultaneously injected to fill the wounds. After surgery, patients were monitored in the intensive care unit for at least 6 h, and fasted for at least 24 h. All patients were administered antibiotics and drugs to inhibit infection and bleeding. Chest and/or abdominal ultrasound scans were performed 2 h and 24 h after the procedures to check for hemothorax, pneumothorax, abdominal bleeding and other complications.

Chemotherapy

Recently, chemotherapy has emerged as a potentially new treatment option for patients with NSCLC after the failure of first-line chemotherapy [1,7,8]. In this study, 140 patients with NSCLC received 4–6 cycles of platinum-based doublet chemotherapy. Of the patients who underwent additional therapies, 65 had prior cryotherapy and 64 received subsequent DC-CIK immunotherapy.

DC-CIK immunotherapy

A total of 85 patients elected to undergo immunotherapy, which involved four sessions of adoptive transfer of DC-CIK cells. The DC-CIK cells were generated following Good Manufacturing Practice (GMP) guidelines [23,49]. Recombinant human granulocyte–macrophage colony-stimulating factor (rhGM-CSF; PeproTech, Rocky Hill, NJ, USA) was injected (150 μg) 24 h before blood collection to mobilize white blood cells.

For culture of CIK cells, PBMCs were suspended in "CIK" medium [X-VIVO 15 (Lonza), 1000 U/ml IL-2 (Peprotech), 2.5 μg/ml monoclonal antibody to CD3 (OKT-3; Jansen-Kyowa, Tokyo, Japan), 25 μg/ml phytohemagglutinin (Peprotech) and 1000 U/ml interferon (IFN)-γ (Peprotech)]. The CIK cells were allowed to grow and then continuously passaged. At approximately 7 days of culture, the CIK cells were passaged to fourteen T225 flasks. Cells adhering to the flasks were removed with a cell spatula, centrifuged and resuspended in "DC-CIK" medium [X-VIVO 15 (Lonza), 400 U/ml IL-2 and 0.5 μg/ml monoclonal antibody to CD3]. All DCs were distributed evenly in the 14 T225 flasks containing CIK cells (approximately 10^8 DCs per flask). After co-culture for 24–48 h, nearly a week after cryosurgery, the DC-CIKs were harvested and suspended in 100 ml saline for intravenous injection (cells were collected on 4 consecutive days; 6×10^9 to 10×10^9 cells were collected on each day). The final cell products were assessed for viability by the dye-exclusion test and checked twice for possible contamination by bacteria, fungi and endotoxins. All cell preparation processes were performed by the same technician, and assessed by another technician; all tests for immune functions were performed by a single technician.

The treatment programs then proceeded as follows: of the 86 patients who opted for cryotherapy as their first treatment, 21 received immunotherapy one week after cryosurgery, and 33 received immunotherapy following cryo-chemotherapy. Peripheral blood (80 mL) was drawn 1–2 days before cryosurgery; cell transfusion (approximately 10^{10} cells each session) commenced 2–4 days after cryosurgery (following nine days of cell culture), and was continued for four days.

To reduce side effects and improve constitution in the 31 patients who opted to undergo immunotherapy after chemotherapy, blood was drawn 1–2 days before the first chemotherapy cycle. After cell culture, the DC-CIK cells were divided into four aliquots (each containing approximately 10^{10} cells) and cryopreserved in liquid nitrogen. Cell transfusion commenced 7 days after the last chemotherapy cycle and continued for 4 days, as described above.

The final cell products, determined by flow cytometry, were as follows: natural killer T (NKT) cells, CD3+CD56+ (27 ± 5%) and CD3+ (87 ± 8%); and natural killer cells (NK), CD3–CD56+

(15 ± 4%). The cells were assessed for viability using the dye-exclusion test, and checked twice for possible contamination with bacteria, fungi or endotoxins.

Evaluation and statistical analysis

Side effects following comprehensive cryoablation were recorded and classified in accordance with the Common Terminology Criteria of Adverse Events (CTCAE) v. 4.0 [2]. The revised Response Evaluation Criteria in Solid Tumors (RECIST; version 1.1) were used to assess intra- and extrapancreatic cancers [9]. Three diagnostic radiologists determined whether or not tumor progression or recurrence had occurred. Overall survival (OS) was defined as the time from diagnosis of metastatic NSCLC to patient death. Kaplan–Meier analysis with Long-rank test was used to analyze OS between patients with or without comprehensive cryotherapy; Bonferroni correction for multiple comparisons was used to analyze OS among five combined therapies. A difference of $P < 0.05$ was considered significant; $P < 0.01$ or $P < 0.001$ were considered highly significant. All analyses were performed using GraphPad Prism v. 5.0 software (GraphPad, San Diego, CA, USA).

Results

Clinical data

A total of 161 patients satisfied the selection criteria and were enrolled in this study, and the basic clinical data were shown as Table 1. Moderate or severe pain in either the primary or metastatic lesions (39 patients), and the use of oral pain medication (62 patients) were common before hospitalization. Metastatic NSCLC was diagnosed in 97 patients from our hospitals; the remainder of the patients were diagnosed at other hospitals and then referred to our hospitals for further treatment. All patients were offered the full range of options by their in-charge doctors, and the patient's choice of treatment options (Fig. 1) depended on economic concerns, their health, and their understanding of the treatment concepts.

Perioperative outcomes

In this study, percutaneous comprehensive cryoablation was successfully performed in 86 patients. There were no deaths associated with the adverse side effects of the cryosurgery, and no severe complications post-procedure, such as cardiac arrest, hepatic failure, renal failure, pathological fracture or respiratory failure. However, transient hemoptysis occurred in 51 patients (59%) during 78 sessions and pneumothorax occurred in 45 patients (52%) during 63 sessions. Both of these complications occurred immediately after completion of the procedure and patients recovered within one week. Bradycardia, hypotension and fever were experienced by seven (8%), 9 (10%) and 21 (24%) patients, respectively, and were resolved following appropriate treatments. Puncture point pains were controlled in all cases by loxoprofen, this was discontinued within one week. A dull pain in the anterior chest was experienced by 23 patients (27%) after treatment, probably caused by damage to the intercostal nerves, and usually resolved naturally within a few weeks. Thrombocytopenia occurred in 14 patients (16%) after 22 sessions, and returned to normal within 8–13 days without requiring treatment. A cough with blood-streaked sputum was reported by 37 patients (43%) after 57 sessions, but improved within 3–5 days without treatment. Within one week of cryoablation therapy, the pain score decreased by over 50% in 55 patients (64%); analgesic consumption decreased by 50% in 52 patients (60%); and the KPS score increased $\geqslant$20 in 61 patients (71%).

None of the 140 patients who received chemotherapy experienced severe bone marrow suppression. Only minor side effects were reported, such as anemia, skin petechiae, fatigue, oral pain, constipation and symptoms of peripheral neuropathy. Relief of these side effects occurred 3–4 weeks after chemotherapy or 1–2 weeks after chemo-immunotherapy. No additional complications or adverse side effects occurred in the 85 patients who received immunotherapy, except for fever, which was experienced after cell transfusion in 24 patients: ~38 °C in 26 cases for 15 patients; and ~39 °C in 14 cases for seven patients. The high temperatures were controlled within 6 h by physical cooling. The remaining two patients experienced fever of up to 40.1 °C, which was controlled within 6 h following an intramuscular injection of Phenergan.

Table 1
Basic clinical data of 161 patients in this study.

		86 Patients with cryotherapy	75 Patients without cryotherapy
Sex	Male	36	31
	Female	50	44
Nationality	China	41	35
	Southeast Asia	24	19
	Middle East	21	21
Pathologic type	Adenocarcinoma	58	51
	Squamous cell carcinoma	28	24
Age (yr)	Average	37–68	38–71
	Range	52	53
Metastatic site	Bone	51 (79 lesions)	42 (68 lesions)
	Liver	22 (34 lesions)	17 (26 lesions)
	Contralateral lung	10 (16 lesions)	8 (11 lesions)
	Other sites	14 (29 lesions)	10 (23 lesions)

Influence of therapies on overall survival

The median OS for all 161 patients up to the final follow-up date was 17 months (95% CI, 15.3–18.8 months). OS of patients who received comprehensive cryotherapy (median OS, 20 months; 95% CI, 20.4–25.7 months; Fig. 2A)was significantly longer than that of patients who did not received cryotherapy (median OS, 10 months; 95% CI, 9.3–11.2 months; $P < 0.0001$). The patients who had undergone cryo-chemo-immunotherapy had the longest survival time (median OS, 27 months; 95% CI, 26.6–37.0 months; Fig. 2B) compared to those underwent cryo-chemotherapy (median OS, 18 months; 95% CI, 14.8–19.2 months; $P < 0.001$) or cryo-immunotherapy (median OS, 17 months; 95% CI, 15.3–22.3 months; $P < 0.001$). Meanwhile, the OS of patients who had undergone combined-cryotherapies was significantly longer than the OS of patients who underwent chemotherapy (median OS, 8.5 months; 95% CI, 7.7–10.4 months;$P < 0.001$) or chemo-immunotherapy (median OS, 12 months; 95% CI, 10.7–13.1 months;$P < 0.001$).

Discussion

Although systemic chemotherapy is the principle treatment for metastatic NSCLC, the prognosis of these patients remains poor [33]; currently a maximum of 4–6 cycles of platinum-based doublets is recommended as standard first-line treatment and the median OS is 8–11 months [41]. In this retrospective study, the median OS was 8.5 months for the 44 patients who undertook platinum-based doublet chemotherapy. It has been suggested that CIK immunotherapy may prevent recurrence and improve quality of life in cancer patients [18]; and that DCs can potentially decrease the proportion of CD4+CD25+ regulatory T cells, which can suppress immune function and increase the populations of

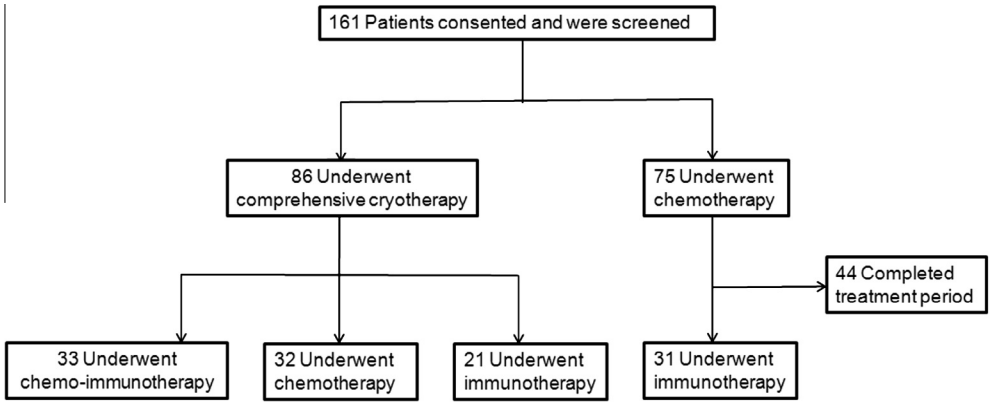

Fig. 1. A tree diagram of the treatment programs selected in this study. The treatment combinations selected by the 161 patients with metastatic NSCLC enrolled in this study, which included cryotherapy, chemotherapy and DC-CIK immunotherapy, are shown.

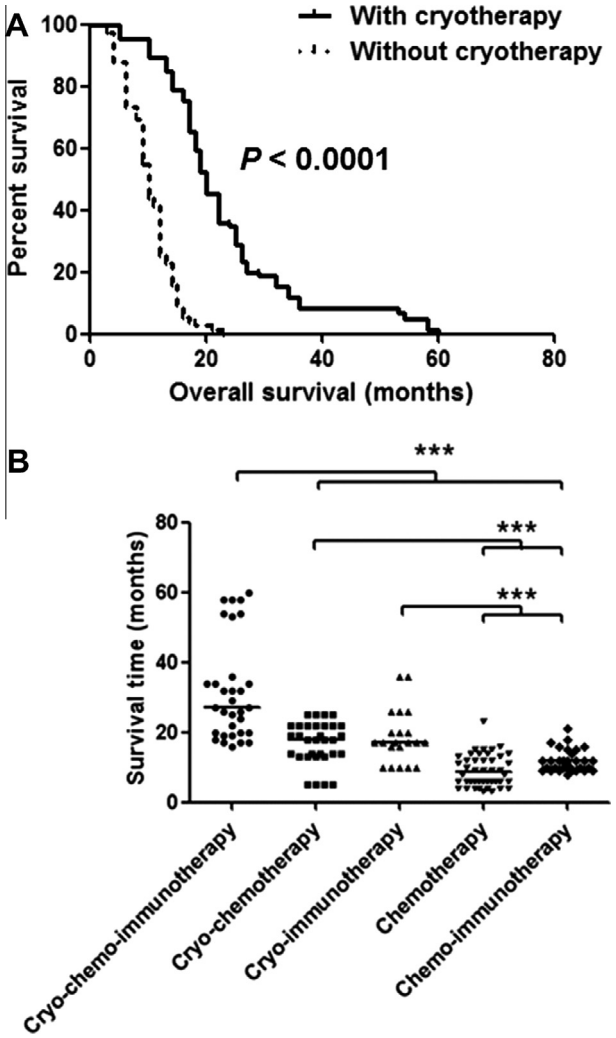

Fig. 2. Analysis of overall survival (OS) for the different treatment options selected in this study. For patients with or with our comprehensive cryotherapy, OS was compared by Kaplan–Meier analysis with Long-rank test (A); for patients underwent five different treatment combinations, OS was compared by Bonferroni correction for multiple comparisons (B). The horizontal lines above the symbols represent median OS; *** represents $P < 0.001$.

CD3+CD56+ NKT cells [14]. DC-CIK immunotherapy was performed after chemotherapy in 31 patients, with the aim of supplementing lymphocyte and improving constitution. Although the median OS in this chemo-immunotherapy group was 12 months, this increase was not statistically significant compared to chemotherapy alone ($P > 0.05$). This result conflicts with several previous studies; for example, Yang et al. reported that chemotherapy plus DC-CIK lead to improved clinical outcomes in patients with advanced NSCLC compared to chemotherapy alone [49]. A possible explanation is that tumors present an organ-like structural environment which is hostile toward immune cells; thereby enabling the tumors to evade immune surveillance [36]. Additional ablation therapy, such as cryotherapy [38] and microwave ablation [51], to disrupt the tumor's organ-like structure may improve the therapeutic effects of immunotherapy in metastatic NSCLC.

Cryosurgery is widely accepted as a minimally invasive curative technique for solid tumors such as prostate cancer, renal cell carcinoma, hepatocellular carcinoma and metastatic liver deposits [22,47], and has recently emerged as a new therapy for lung cancer [30]. In addition to treating a tumor in single organs, cryotherapy can be used to treat multiple lesions simultaneously (e.g. comprehensive cryoablation in metastatic pancreatic [4], hepatic [27] and breast cancers [28]), with similar reported side effects to those found in this study. In order to increase the rate of cell death in the peripheral cryolesion, adjunctive therapies (including transcatheter arterial chemoembolization [48], chemotherapy [31], brachytherapy [4] or immunotherapy [32]) is required. Previous investigations, both *in vitro* [26] and *in vivo* [13,21], have shown that a combination of cryosurgery with chemotherapy can enhance cell death due to necrosis and apoptosis and significantly decrease tumor volume, compared to cryosurgery or chemotherapy alone [10–12]. The data from our study supported these findings, by showing that a combination of cryosurgery with chemotherapy or cryo-immunotherapy significantly improved OS, compared to chemotherapy or chemo-immunotherapy alone. Adoptive transfer of CIK cells with DCs has been shown to be effective when the tumors are ablated by cryosurgery: cryosurgery not only breaks down the tumor structure; it also attracts phagocytic cells (DCs and macrophages), which remove the dead and apoptotic cells and cross-prime CD4+ and CD8+ T lymphocytes. This is known as the cryo-immunological response [39,42,45], by which cryo-immunotherapy has been prove to be a very important way of improving OS [46].

There is increasing evidence from both clinical trials and animal studies to suggest that adoptive immunotherapy can reduce the rates of recurrence and metastasis in malignant tumors [37]. These treatments have cytotoxic effects against several different types of tumor cells, such as Hodgkin disease, non-Hodgkin lymphoma, and hepatocellular carcinoma [50]. Cryosurgery can induce necrosis or apoptosis of tumor cells; chemotherapy will increase cell death induced by cryosurgery; and DC-CIK immunotherapy can potentially

kill any residual chemotherapy-resistant tumor cells (e.g. cancer stem cells) [3,40] and enhance recovery from the side effects of chemotherapy. Our results supported this evidence by showing relief from multiple side effects 3–4 weeks after chemotherapy reduced to 1–2 weeks after chemo-immunotherapy. Making full use of the synergy among many effective methods, cryo-chemo-immunotherapy may combine the strengths of local and systemic therapies to achieve the best therapeutic outcome and increase OS. As tumors are heterogeneous [16], and metastasis and chemo-resistance are characteristics of cancer stem cells [35], we suggest that a combination of multiple therapeutic approaches may be the most effective way to treat metastatic NSCLC.

In summary, this retrospective study conducted a preliminary investigation into the therapeutic effects of combined cryosurgery, chemotherapy and DC-CIK immunotherapy in patients with metastatic NSCLC. Our findings showed that a combination of all three therapies was the best option for improving OS. However, these treatment options were only available to patients with metastatic NSCLC who met the filtering criteria, and who were therefore in better physical condition compared to patients who were excluded. Treatment options for patients with poor physical fitness will be different; therefore, our conclusions need to be confirmed in a larger cohort of patients, and further study is required.

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