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Exploratory Clinical Safety and Efficacy Study of Adoptive Transfer of Tumor Infiltration Lymphocytes in Patients with Refractory Malignant Ascites

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Abstract: Objective: To study the safety and clinical efficacy of intraperitoneal transfer of autologous tumor infiltrating lymphocytes (TIL) in the treatment of recurrent and refractory malignant ascites. Methods: A single-arm, open-label study, 9 patients with previously unsatisfied ascites treated had been enrolled to receive both intraperitoneal and intravenous or intraperitoneal alone transfer of TILs in Beijing Zhongguancun Hospital, Chinese Academy of Sciences from December 2019 to October 2021, and the complete record including efficacy and adverse reactions of the treatment were observed. Results: Of those nine patients, including 1 case of liver cancer, 1 case of esophageal cancer, 1 case of lung cancer and 1 case of ovarian cancer, 2 cases of rectal cancer and 3 cases of gastric cancer, all with multiple site metastases. Six patients (66.7%) were diagnosed with omental, mesenteric or peritoneal metastases by CT. Both pleural effusion and ascites were found in 8 cases (88.9%). The median maximum fluid depth of peritoneal effusion was 7.8 cm (3.9-12cm) and the median maximum fluid depth of pleural effusion was 6.2cm (0.1-12.8cm) measured by ultrasonography. All patients received other treatments before TILs transfer: intraperitoneal chemotherapy in 7 cases (77.8%), peritoneal drainage in 6 cases (66.7%), and systemic chemotherapy in 4 cases (44.4%). TIL transfer was performed 19 times, including 11 times of intraperitoneal and 8 times of intravenous transfer. One patient had fever (39.3°C) and chills during intravenous transfer, which was relieved after intravenous injection of dexamethasone. Median survival time of patients at post- adoptive transfer was 12.3 weeks (0.5-29.2 weeks), and one patient was still alive at 29.2 weeks. Conclusion: Most patients with malignant ascites have multiple metastases and pleural effusion. Combined with supportive treatment, multidisciplinary comprehensive treatment can still be carried out. After proper screening for patients, the success rate of preparing TILs from malignant ascites was high, and the completion rate of transfer was high. The adverse reactions of intraperitoneal and intravenous transfer were minimal. Further study of treatment efficacy is warranted.

Keywords: Ascites, Peritoneal Metastasis, Tumor Infiltrating Lymphocytes, Adoptive Cell Transfer, Adverse Event

1. Introduction

The use of tumor infiltrating lymphocytes (TIL) in the treatment of tumors started from malignant melanoma. Goff et al. found that T cells could be cultured from resected melanoma metastases, and if they were cultured together with IL-2, more than 2/3 of fresh specimens could be cultured with T cells that recognized their own tumors [1]. In 1994, Rosenberg et al. reported the efficacy of TIL in the treatment of malignant melanoma. They selected patients with malignant melanoma with measurable metastases, and

patients were treated with TIL and systemic IL-2 only. The median number of cell transferred were nearly 2×10^{11}. The overall objective response rate was 34%, and 7% of patients had tumor response duration for more than one year. Rosenberg followed up with a series of improvements in TIL preparation and transfer, and the objective response rate for patients with metastatic malignant melanoma is now as high as 54%, with 20% of patients having a durable complete response after 5-8 years of follow-up [2].

The adoptive transfer of TIL has a relatively good safety record, and the most common side effects are associated with treatments that are used together with TIL transfer, including high-dose IL-2 injections and chemotherapy [3]. Leukopenia, including neutropenia, lymphopenia, and long-term suppression of CD4+ T cells, occurs in almost all patients receiving lymphocyte-clearing chemotherapy. Nonhematologic toxicities associated with lymphocytic clearance chemotherapy include diarrhea, hyperbilirubinemia, and fludarabine-induced neurotoxicity. Opportunistic infections may occur in a small number of patients. The high-grade toxicity associated with TIL transfer is rare and often difficult to distinguish from the residual IL-2-related reactions in the TIL preparation, such as fever, hypotension, and dyspnea [4]. Since most patients with malignant pleural effusion were in the end-stage of tumor and could not tolerate the toxic side effects of high-dose IL-2 or lymphocyte clearance chemotherapy, TIL transfer plus low-dose IL-2 injection was used [5] and the safety and efficacy of this treatment method were observed.

2. Materials and Methods

2.1. Patients' Enrollment

This study was an open-label, single-arm, feasibility study (Project of Wu Jieping Foundation, No. 320.6750.2020-8-2) for patients with advanced malignant ascites. It was approved by the Ethics Committee of Beijing Zhongguancun Hospital and recorded in the Medical Research Registration Information System of the National Health Commission.

Inclusion criteria: 18-95 years-old with malignant ascites; diagnosed by pathology and/or cytology; unable to accept or unwilling to accept other treatments; the amount of ascites confirmed by imaging examination was more than 500ml; ECOG score was 0-4; the expected survival time was >2 weeks; major organ function was acceptable, that is, the relevant examination indicators met the following requirements within 14 days before enrollment: blood routine: hemoglobin >70g/L, neutrophil $>1.5\times10^9$/L, platelets $>50\times10^9$/L, serum aspartate aminotransferase (AST), alanine aminotransferase (ALT)<5×ULN (upper limit of normal value), serum total bilirubin (TBil)<2×ULN, serum creatinine level (Scr)<2×ULN, and left ventricular ejection fraction >50% (Echocardiographic Doppler assessment).

Exclusion criteria: pregnant or lactating women; uncontrolled infectious diseases; with organ transplants or on immunosuppressive agents; with severe autoimmune diseases; severe infectious diseases, including abdominal infections or infectious peritonitis; allergic to the biological agent used in this treatment; with active bleeding; with a history of psychotropic drug abuse or with mental disorders; had participated in clinical trials of other treatments within four weeks.

A total of 9 patients were enrolled from December 2019 to October 2021.

2.2. Treatment Methods

After admission, the patients were tested for ascites and were given nutritional support. Diuretics were used and water and electricity balance were maintained. The daily urine volume was kept at more than 2000ml. Imaging examination was performed 3 days before the scheduled TIL transfer, and the volume of thoracoabdominal water was found to be more than 500ml. TILs were transferred with 200ml saline twice: once intraperitoneal and once intravenous. Cell transfer was finished in about 30 minutes. The interval between the two transfer was one day. Prophylactic intramuscular injection of phenazine 25mg or chlorpromazine 25mg was used before transfer. Other treatments were unchanged around cell transfer.

2.3. TIL Preparation

Ascites 500ml to 1000ml were drawn. RetroNectin® and anti-CD3 mab (both provided by Takara, Japan) were used as cell culture stimulators, and GT-T551 H3 serum-free medium (Takara, Japan) was used as cell culture medium and prepared according to its usage procedure. RetroNectin® contains the cell attachment domain, heparin binding domain and CS-1 site of human fibronectin. The molecular weight of RetroNectin® is about 63K, and its main physiological functions include involvement in cell attachment, extension, differentiation and proliferation [6].

The specific preparation process was as follows. About 500-1000ml of ascites or pleural effusion was collected and centrifuged, the supernatant was removed, the cells were resuspended with PBS, and the cell suspension was added to the lymphocyte separation solution and centrifuged. After centrifugation, the lymphocytes in the tunica albuginea layer were collected and rinsed 3 times with PBS. After centrifugation, PBS supernatant was removed, and serum-free medium was added to prepare the cell suspension. The prepared cell suspension was added to the culture flask coated with RetroNectin® and anti-CD3 mab, and placed in carbon dioxide incubator (SANYO Company, Japan) at 37°C for cell culture. On the 4th day of culture, the lymphocytes were transferred to the culture bag (provided by Takara, Japan) for further expansion of culture. The status of cell culture was observed every day during the process, and fresh serum-free medium was supplemented every 2-3 days. On the 14th day of culture, the cells were harvested by centrifugation, rinsed 3 times with normal saline, and resuspended with normal saline to prepare the cell products. Bacteria and mycoplasma culture and endotoxin examination

were performed before transfusion, and lymphocyte subtype was detected by flow cytometry.

2.4. Cell Surface Markers Detection by Flow Cytometry

Cell products were sent to Beijing Adicom Medical Laboratory Center for flow cytometry detection. The method is as follows. Appropriate amount of cell suspension is taken, and about 5×10^5 cells/tubes are added to flow detection tubes and centrifuged at 1000rpm for 5 minutes. Discard the supernatant, then add 1ml PBS to each tube, and wash twice. Discard the supernatant, and the fluorescent antibody labeling of the surface antigen to be tested (CD3/CD4/CD8, Agilent company, USA) is added, 10μl each, shake and mix well, and place in the dark for 15min at room temperature. The cells are washed by adding 1ml PBS and centrifuged at 1000rpm for 10 minutes. Discard the supernatant, add 1ml PBS to each tube, shake and mix well, and measure by flow cytometry.

2.5. Study Endpoints

Clinical efficacy was evaluated by hematology and imaging method within 1 day to 8 weeks after TIL transfer. The primary end point was treatment feasibility, defined as completion of TIL therapy without early discontinuation because of unacceptable adverse events. Secondary end points included the use of Common Terminology Criteria for Adverse Events (CTCAE V.5.0) [7] to assess clinical safety, clinical response, objective response rate (ORR) based on Response Evaluation Criteria in Solid Tumors (RECIST V1.1), overall survival (OS), and progression-free survival (PFS). OS was defined as the time from the start of treatment to death, and PFS was defined as the time from the start of treatment to the first progression or death from any cause. The current paper focused on treatment feasibility and clinical safety.

2.6. Statistical Analysis

IBM SSPS software version 25.0 was used for statistical analysis. Chi-square test or exact probability method was used for categorical data analysis, and Kaplan-Meier method and log-rank test were used for survival analysis. $p<0.05$ was considered statistically significant.

3. Results

3.1. Patient Characteristics and Completion of TIL Cell Transfer

Nine patients with malignant ascites who were hospitalized in the Department of Oncology, Zhongguancun Hospital, Chinese Academy of Sciences from December 2019 to October 2019 were selected to participate in the clinical feasibility study of TIL cells peritoneal/intravenous transfer. Among them, there were 1 case of liver cancer, esophageal cancer, lung cancer, ovarian cancer, 2 cases of rectal cancer, and 3 cases of gastric cancer. All patients had multi-site metastasis. Six patients with omentum, mesentery or peritoneum metastasis were diagnosed by CT, including 5 patients with gastric cancer and rectal cancer, and 1 patient with lung cancer. Except for the patient with hepatocellular carcinoma, the other 8 patients presented with both pleural effusion and ascites, and one of them also had pericardial effusion. The median maximum depth of fluid under ultrasound was 7.8cm (3.9-12cm) in peritoneal effusion and 6.2cm (0.1-12.8cm) in pleural effusion. All patients received other treatments before TIL transfer, including 7 cases of thoracoabdominal cavity chemotherapy, 6 cases of thoracoabdominal puncture drainage, and 4 cases of systemic chemotherapy. The nine patients received TIL transfer 19 times, including 11 times of intraperitoneal transfer and 8 times of intravenous transfer. The details are shown in the table below (Table 1).

Table 1. *Basic Patient Characteristics.*

Serial number	gender	age	Primary tumors	Transfer area	Maximum fluid depth of ascites (cm)	Maximum fluid depth of pleural fluid (cm)	Times of cell transfer	Method of infusion
1	male	56	liver	lung	7.8	NA	2	Abdominal cavity 2 times
2	male	47	The stomach	Peritoneal liver	10.4	Left 5.4 2.7 right	2	Abdominal cavity once. Intravenous once
3	male	58	The rectum	Abdominal wall greater omentum inguinal lymph nodes	3.9	Left 8.2 2.7 right	3	Abdominal cavity once. intravenous 2 times
4	male	59	The rectum	Pelvic liver omentum mesentery	5.9	Left 7.2	2	Abdominal cavity once. intravenous once
5	male	54	The esophagus	Pleural and peritoneal pericardial effusion, in which cancer cells were detected	6	Left right 10.5 10.7	2	The thoracic cavity once. intravenous once
6	female	46	ovary	Vaginal stump liver lung pelvic abdominal lymph nodes	12	Left 5.1 6.2 right	2	Abdominal cavity 2 times
7	male	67	Right lung	Brain metastases bone retroperitoneal abdominal lymph nodes greater omentum	10.9	Left 1.1 12.8 right	2	The thoracic cavity once. intravenous once
8	female	54	The stomach	Thickening of peritoneum and omentum in the appendix area of the fossa ileocecum of both ovaries	6.3	Left 6.0	2	intravenous once. Abdominal cavity 1 time
9	female	45	The stomach	Omentum ovarian peritoneum	11.6	Left a small amount of	2	intravenous once. Abdominal cavity once.

3.2. Hematological Indicators

All patients underwent hematologic tests prior to treatment, and the results are shown in the table below. Two patients (22.2%) had elevated leukocytes (normal values 3.5 to 9.5 x 10^9). Seven patients (77.8%) had decreased lymphocyte ratio (normal value 20-50%). Five patients (55.5%) had decreased serum albumin (normal value 35-55g/L). All 9 patients had normal serum globulin (normal value 20-45g/L), and 3 patients had mild hyponatremia (normal value 135-145mmol/L). There were 2 cases of hypokalemia (normal value 3.5-5.5mmol/L), 7 cases (100%, 7/7) with elevated serum il-6 level (normal value <7pg/ml), 2 cases (normal value <300pg/ml) with elevated pro-BNP level, and 4 cases (50%, 4/8) with decreased T3 level (normal value 1.3-3.1nmol/L). Details are provided in the table below (Table 2).

Table 2. *Hematological Indicators.*

Serial number	WBC	Percentage of lymphocytes (%)	A	G	Na	K	IL-6	Pro-BNP	T3
1	3.99	9	32.5	30.8	136	3.8	48.6	44.7	1.43
2	14.92	9.2	32.2	37.6	133	4.2	72.37	71.2	1.5
3	7.09	53.2	36.1	24.8	134	4.2	34.96	24.4	1.05
4	12.22	8.3	35.6	28.2	139	3.6	22.67	149.9	0.98
5	7.45	11.5	31	22.5	137	3.5	49.69	3851	0.84
6	4.03	7.9	35.6	23.1	134	3.2	36.83	41.3	NA
7	5.05	11.9	36.3	23.9	141	3.3	NA	79.5	NA
8	3.71	15.4	27.2	25.9	135	3.7	57.74	453	1.38
9	4.64	28.9	28.2	21.1	137	3.5	NA	35.6	0.97

3.3. Characteristics and Adverse Events of TIL

TIL was successfully prepared in all patients (9/9, 100%) and passed tests for sterility and cell viability. Flow cytometry results showed that the median number of cells in TIL prepared from malignant ascites was $143X10^8$ ($127-224X10^8$), with CD3 +, CD4 + T cells in the proportion of 34.1% (21.9%-46.7%), CD3 + CD8 + T cells in a proportion of 55.4% (30.6%-69.2%). The median IL-2 reinfusion volume was 70,000 U (4-125,000 U). Grade I adverse events occurred in 1 patient half an hour after the end of intravenous lymphocyte infusion. Symptoms included chills, fever with temperature up to 39.3°C. No decrease of blood pressure was observed and body temperature went down to 36.7°C after intravenous infusion of 5mg of dexamethasone. The incidence of adverse events was 11.1% (1/9) per person and 5.3% (1/19) per number of reinfusion, including 0 for intraperitoneal reinfusion and 12.5% (1/8) for intravenous reinfusion. The median survival time was 11.9 weeks (0.5-29.2 weeks) after the first infusion (Table 3). One patient with ovarian cancer received intraperitoneal infusion of chemotherapy drugs plus TIL, and was still alive at 29.2 weeks.

Table 3. *Characteristics and Adverse Events of TIL treatment.*

Serial number	Treatment before cell reinfusion	Number of transferred cells ($\times10^8$)	CD3+CD8+ Percentage (%)	CD3+CD4+ Percentage (%)	Infusion of IL-2 (ten thousand IU)	Adverse events within 72 hours after infusion	Survival time after TIL transfer (weeks)
1	The abdominal cavity drainage	160	69.2	24.3	4	NA	4
2	Systemic chemotherapy Abdominal cavity perfusion	151	68.8	34.1	7	NA	17.5
3	Abdominal cavity perfusion	224	65.7	21.9	8	NA	2.6
4	Systemic chemotherapy The abdominal cavity drainage	145	55.4	44.1	12.5	NA	11.9
5	Chest drainage Thoracoabdominal perfusion	127	47.2	46.7	7	NA	0.5
6	The abdominal cavity drainage Abdominal cavity perfusion	135	51.8	46.1	4	NA	29.2
7	The chest cavity is drained and the abdomen is perfused Intravenous chemotherapy, targeted therapy	137	30.6	30.0	7	NA	12.3
8	Abdominal cavity perfusion	142	51.9	29.9	10	Moderate *	17.6
9	The abdominal cavity drainage Metronomic chemotherapy, intraperitoneal perfusion	143	66.1	34.1	4	NA	25.7

4. Discussion

Malignant ascites associated with tumor is a common clinical complication. Local injection of cytokines, talcum powder, and chemotherapy drugs are routinely used in clinical practice. The clinical control rate of malignant ascites is about 20%. He et al. injected autologous cultured CIK cells

locally, and the efficacy rate of clinical control reached 60% for refractory pleural effusion of epithelial-derived tumors with positive cytology test [8]. Cancer consists of many cancer cells with different life spans that can mutate multiple times, so that the tumor antigen specificity of cancer cells changes at different stages. This is different from other adoptive immunotherapies (e.g., cancer vaccines, antibodies) in which tumor antigens are prespecified [9, 10]. TIL is a highly individualized tumor-specific lymphocyte therapy, which is mainly based on the infiltrating T cells in the patient's own tumor tissue and naturally recognizes a variety of tumor-specific antigens unique to the patient. Secondly, after stimulation with tumor antigens in vivo, TIL is often composed of effector memory T cells, which express chemokine receptors such as CCR5 and CXCR3 on the cell surface, and can be easily localized to tumor tissues after being transferred to the patient. Third, during the early development of T-cell immunity, TIL shows negative selection for TCR and does not have the on-target or off-target effect. Therefore, TIL transfer therapy does not produce cross-reactivity with normal tissue antigens as in engineered T cells due to affinity enhancement of TCR. T cells grown in the tumor microenvironment are not potent enough to generate an adequate antitumor immune response due to T cell depletion caused by immunosuppression. Separation of TIL cells from fresh tumor tissues with in vitro amplification avoids the tumor immunosuppression environment, and can produce highly individualized and tumor specific, and highly activated autologous T cells. The preparation cost will be much lower than engineered T cells, with much smaller side effects, and enhanced antitumor immune effects.

Considering that about 20-40% of patients cannot have their TIL cells successfully expanded in vitro from their tumor tissue, and that patients with malignant ascites or pleural effusions are at risk if receiving tumor biopsy, the technical path of extracting and expanding lymphocytes from ascites or pleural effusions was adopted. The latter approach has the following advantages over extracting TIL from fresh tumor specimens [11]. Firstly, the number of T cells in ascites or malignant pleural effusion was higher and the culture time was shorter. Secondly, T cells in malignant ascites or pleural effusion generally represent the characteristics of all types of TIL cells. TIL extracted from malignant ascites of pleural effusion can secrete more IFN-γ and TNF-α, and have a strong ability to lyse target cells. A report from Radboud University Medical Center in the Netherlands showed that 100% of ovarian cancer patients with ascites could produce T cells from ascites. CD4+ T cells accounted for 45.5% (2.0-78.0%) (including Th and Treg cells) and CD8+ T cells accounted for 33.0% (1.0-65.0%) in CD3+ subsets [12]. The reported methods for in vitro expansion of tumor-infiltrating lymphocytes from ascites or pleural effusion were cumbersome and time-consuming, and the proliferation effect is often only up to 10^8- 10^9A cell [13]. RetroNectin® is a recombinant human fibrin fragment originally used in the field of gene transfection, which was later found to be effective in improving the proliferation efficiency of lymphocytes [14]. In this study, RetroNectin® was used as a stimulator to induce T lymphocytes in culture [6]. The success rate of TIL preparation from malignant ascites was 100%, and the number of T cells produced could be more than 10^{10}. The proportion of CD3+CD4+T cells in TIL was 21.9%-42.3%, and the proportion of CD3+CD8+ T cells was 30.2%-69.2%. The number of CD3+CD8+ T cells in TIL was higher than that of CD3+CD4+T cells in each patient.

Clinical efficacy of therapy with transfer of TIL cells that was correlated with that the cells transferred could survive long enough in the body of the patient. In one study, tumor-specific TIL cells were detected in peripheral blood of patients up to 34 months after infusion [15]. In another clinical study, an increase in the effective response to TIL infusion was associated with an increase in the half-life of tumor-specific TIL clones (CR, 132-173 days, PR, 31-53 days, NR, 13-15 days, $P < 0.05$) [16]. Multiple clinical trials of CAR-T infusion in the treatment of tumors are ongoing, and it is interesting to note that CAR-T cells cannot be found in intraperitoneal tumors after intravenous CAR-T administration, whereas they can be found in intraperitoneal tumors after intraperitoneal CAR-T infusion [17]. Katz et al. found that anti-CEA CART mice had a 37-fold increase in tumor shrinkage when injected intraperitoneally versus intravenously [18]. The intraperitoneal injection of CAR-T cells expressing TAA gene avoids the "on target off tumor" immune effects caused by intravenous administration, avoids cytokine release syndrome, and significantly reduces neurotoxicity [19]. Similarly, intraperitoneal administration of cytokines not only participates in the regulation of the immunosuppressive environment in the abdominal cavity, but also presents fewer systemic side effects and longer intracavitary drug concentrations. For example, when intraperitoneal administration of IFNs, at 5×10^6 to 15 x 10^6 units, the intraperitoneal concentration is 30-200 times that of concentration in peripheral blood and the half-life is significantly prolonged (10-32 hours for intraperitoneal administration and 5-13 hours for peripheral intravenous administration) [20]. If Il-2 was injected into the thoracic cavity, and the local concentration of IL-2 was 6000 times higher than that in the peripheral blood [21]. Local injection of IL-2 (including intraperitoneal administration) has been shown to have a clear effect on a variety of human malignant tumors, and the effect is better than that of intravenous administration. Besides, some tumor patients treated with local injection of IL-2 also have acquired tumor immunity. But when IL-2 was administered locally and the total dose was more than 10^8 unit, the same side effects as in systemic IL-2 administration could be observed. Therefore, it is not advised to combine high-dose of IL-2 in the thoracic and abdominal transfer of immune cells [22].

To deal with the immunosuppressive environment in the abdominal cavity, ascites drainage, intraperitoneal infusion chemotherapy, and systemic beat-chemotherapy were performed. Those treatments did not increase the rate of

adverse events of patients. In this study, only 1 patient (11.1%) had grade I adverse events (fever and chills) within 72 hours after TIL transfer. The rate was similar to that of reports from domestic and foreign literatures [23].

5. Conclusion

This study showed that the preparation of TIL from malignant ascites, combined with adoptive intraperitoneal and intravenous infusion with low-dose IL-2, had a high success rate, good safety record, and a median survival time of nearly 3 months for patients with end-stage malignant ascites. This treatment method is worthy of further clinical studies.

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