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ORIGINAL PAPER

Effect of Transcatheter Arterial Chemoembolization Combined with Argon–Helium Cryosurgery System on the Changes of NK Cells and T Cell Subsets in Peripheral Blood of Hepatocellular Carcinoma Patients

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Abstract Hepatocellular carcinoma (HCC) is one of the most aggressive tumors in humans. T lymphocytes and natural killer (NK) cells are the body's first line of defense to prevent tumor cell growth. Previous studies have demonstrated that transcatheter arterial chemoembolization (TACE) combined with argon–helium cryosurgery system (AHCS) can effectively treat liver cancer. However, the mechanism of the treatment is unclear yet. In the current study, we investigated the effects of TACE combined with AHCS on the changes of T cell subsets and NK cells in peripheral blood of HCC. Our data show that alpha-fetoprotein (AFP) levels in peripheral blood were significantly up-regulated in HCC patients before treatment when compared with healthy people and reduced after TACE combined with AHCS treatment ($P < 0.01$). In addition, we found that CD4+ cells and NK cells decreased ($P < 0.05$) and CD8+ cells increased ($P < 0.05$) in HCC patients when compared with healthy people. After treatment, the CD4+ cells, CD4+/CD8+ ratio, and NK cells were dramatically increased in HCC patients ($P < 0.05$). In contrast, CD8+ cells were significantly decreased ($P < 0.05$). TACE combined with AHCS treatment significantly prolonged 1-year survival rate of HCC patients and did not show significant side effects. Taken together, our data indicate that TACE combined with AHCS treatment improves patients' immune system. It is a feasible and effective therapeutic method for HCC patients.

Keywords TACE · AHCS · T cells · NK cells · Peripheral blood · Hepatocellular carcinoma

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Introduction

Hepatocellular carcinoma (HCC) is one of the common malignant tumors. Operation resection is still the preferred treatment for early HCC [1]. However, HCC patients after surgery have decreased immune function, damaged liver function, metastasis, and recurrence [2–4]. It has been reported that HCC can be treated by transcatheter arterial chemoembolization (TACE) via superselective segmental embolization [5]. However,the treatment strategy has some drawbacks [6]: incomplete embolism and tissue necrosis after surgery [7], collateral circulation quickly occurs after embolization of tumor supplying artery, which is not up to long-term blocking vascular [8], and multiple TACE treatment damages liver function and also leads to liver fibrosis [9]. We therefore sought to find more effective treatment strategies. Since argon–helium cryosurgery system (AHCS) could effectively kill tumor cells [10], in this study, we further explored the anti-tumor effect by combining AHCS with TACE.

AHCS blocks microcirculation through forming thrombosis in small blood vessels, which causes local tissue ischemia and hypoxia, and eventually leads to tumor cell necrosis [11]. AHCS can effectively kill tumor cells in the freezing zone and protects normal liver tissues to the utmost extent [12]. Under ultra-low temperature, tumor cells will be degenerated, dehydrated, carbonized, and they die in minutes [13]. Tumor cell-specific antigen will be formed by absorbing necrotic cell debris in weeks, stimulating the proliferation of cytotoxic T lymphocytes and dendritic cells, and provoking body to generate specific anti-tumor immunological reactions and inhibit growth and recurrence of tumor. Follow-up study has shown that the survival rate of freezing treatment is almost equal to hepatic resection, but the life quality of survival patients is clearly improved.

In current study, we treated HCC patients with AHCS, TACE, and a combination of AHCS with TACE, respectively. To investigate the effects of combination of AHCS with TACE on the treatment of HCC and that when performed alone, the changes of alpha-fetoprotein (AFP), CD3, CD8 (Ts), CD4 (Th) and CD4/CD8 (Th/Ts), and the natural killer (NK) cells were determined before and after treatment.

In addition, treatment of HCC had achieved significant short-term effects, but evaluation standard of curative effect has not been established yet. CT examination is mainly used to determine postoperative complications and identify residual tumor area, which helps direct following treatment [14]. Postoperative digital subtraction angiography (DSA) detects the pathological blood vessels and the number of tumors that are stained [15]. Ultrasonography is more commonly used in clinical, but it is difficult to distinguish residual tumor tissue from coagulative necrosis tissue. Therefore, we used AFP as a detection index for primary HCC to find a suitable evaluation standard for primary HCC therapy. Our study provides a new idea for the treatment of human HCC.

Materials and Methods

Patients

The patients were selected based on the following criteria: (1) All the cases were confirmed by imaging text and pathologic examination, such as B-ultrasound and CT, and primary liver cancer was diagnosed according to the diagnostic criteria of HCC. (2) Patients were $\geq$18 years old. (3) Patients' living conditions were scored according to ECOG. (4) Child classification of liver function was A–B. (5) Patients did not have coagulation disorders, or heart, lung, and kidney dysfunction, or seriously active infection. (6) Patient's life expectancy was more than 3 months. 163 Patients admitted to the hospital during August 2013 and May 2014 were selected according to the criteria. There were 119 males and 44 females. Their age ranges from 28 to 68 years, with average age being 48.0 $\pm$ 0.4 years. 127 cases had primary liver cancer and 109 cases (85.8 %) had AFP levels higher than 400 g/L. The patients were divided into five groups including the healthy group (36 cases), the untreated group (18 cases), the TACE group (36 cases), the AHCS group (32 cases), and the TACE combined AHCS group (41 cases) according to the patient's condition and treatment.

Transcatheter Arterial Chemoembolization Surgery (TACE)

Routine examination was performed before operation, including blood tests (WBC $>4 \times 10^9$/L, and platelets $>80 \times 10^9$/L), liver function tests, alanine aminotransferase measurement (ALT <120 U/L), renal function test, coagulation inspection, and AFP detection. Patients were water fasting for 4–6 h before surgery.

Surgical methods: femoral artery puncture was performed using a modified Seldinger method. Angiography was performed with Cook catheter in the opening of hepatic artery. Chemotherapy drugs (epirubicin, and fluorouracil or cisplatin) were then injected slowly after the size and location of tumor were clarified. Then superselectively, along the catheter drug was slowly injected into ultra-iodized oil emulsion 10–30 mL (epirubicin and lipiodol) to embolize tumor peripheral vascular after tumor-feeding artery was found. To completely block tumor blood supply, patients were supplemented with gelatin sponge particles for embolization when necessary. After conventional lower limb brake for 24 h, supportive treatment was applied to liver and gastric mucosa. Abdominal CT, AFP levels, and liver and kidney function were reviewed after surgery every 4 weeks, for a total of 2–3 times.

Argon–Helium Cryoablation System

Patients were selected for catheter-based cryoablation if their lesions were detected with B-ultrasound or CT, and the puncture was easily located. Patients were excluded if their tumors were close to diaphragm, biliary tract, and blood vessels. American Superconductor argon–helium surgical system equipped with 2-, 3-, 5- and 8-mm freezing probes (USA Enclocare company) was used.

Surgical methods: With the patients under local anesthesia by 1 % lidocaine, puncture points were determined by B-ultrasound or CT. Patients' skin was cut to about 0.8 cm. Needles with core were guided by B-ultrasound in the intercostal or subcostal area. Inner core was removed after needles were punctured into the bottom of the tumor. Needles were removed after introduction of a metal guidewire. The core was extracted after percutaneous introduction of a guidewire into the sheath sets. Cyroprobe was inserted into tumors along puncture sheath and fixed. Puncture sheath of 3–5 cm was pulled out based on the tumor size. Different angles of the needle were operated as described above. Then computer was open with cryoablation system. Temperature of the tip was reduced from −120 to −140 °C within 1 min. Then the tip was froze continuously for 15–20 min and made hockey 1 cm bigger than the size of the tumor margin. The heating system of helium gas was turned on until the temperature was above 30 °C. Then the above cycle was repeated again, bled with gelatin sponge and biological glue packing tract.

Comprehensive treatment strategy: 41 patients were treated with TACE for 1–3 cycles. Many Lipiodoldeposited tumors were detected by CT scans. Cryoablation was conducted 1 month later.

Evaluation Criteria for Treatment Efficacy

According to the results of unenhanced and enhanced CT examination, treatment efficacy was determined based on the disease reduction, the lesion strength, and its size range. Necrosis was classified as complete, incomplete, and partial necrosis. Complete necrosis does not show enhanced lesions or DSA enhancement. Incomplete necrosis shows enhanced lesion, excluding abnormal perfusion and surrounding inflammation after tumor cryotherapy. The rate of tumor necrosis is between 90 and 99 %. The rate of partial necrosis was between 50 and 89 %. The primary tumor is increased or new lesions are discovered within 3–6 months, which indicates tumor recurrence.

Statistical Analysis

SPSS15.0 software was used for statistical analysis. ANOVA was used for mean analysis. Living conditions were analyzed using LogRank method. $P < 0.05$ was considered as statistically significant.

Results

Treatment of HCC Patients with Cryosurgical Ablation Combined with TACE Significantly Reduces AFP Level

Since AFP level is usually up-regulated in HCC patients, it can be used for diagnosis and prognosis of primary liver cancer, which has a significant prognostic value and clinical significance. The differential AFP expression may reflect the differentiation degree of liver cancer cells. In this study, 80 cases from 127 HCC patients had AFP > 400 ng/ml and another 21 HCC patients had AFP > 1000 ng/ml. There were 32 cases in the TACE group, which includes 29 cases with abnormal AFP level. AFP decreased 50 % in 19 cases (65.51 %) after treatment. There were 31 cases in the AHCS, which includes 27 cases with abnormal AFP. AFP decreased 50 % in 18 cases (66.66 %) after treatment. There were 35 cases in the AHCS combined with TACE group, which includes 32 cases with abnormal AFP level. AFP decreased 50 % in 27 cases (84.38 %) after treatment. The results indicate that AHCS combined with TACE treatment is better than single AHCS and TACE treatment. The difference is statistically significant ($P < 0.01$) (Fig. 1).

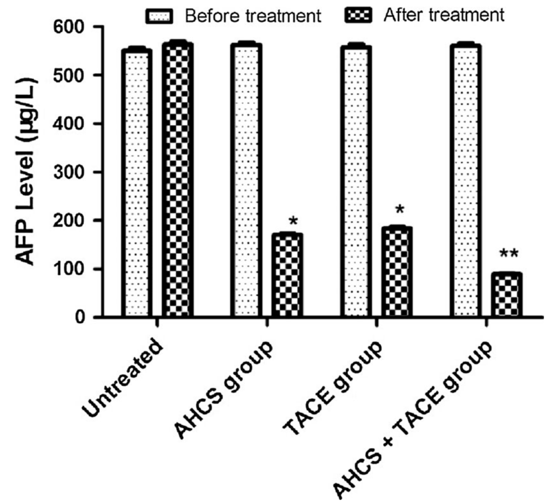

Fig. 1 The AFP levels in each group before and after treatment. The *asterisks* indicate statistically significant difference

Reduced T Lymphocytes and NK Cells in Peripheral Blood in HCC Patients

Since T lymphocytes and NK cells serve as the first line of defense of human body against tumor cell growth, we measured the change in T lymphocytes and NK cells in HCC patients. As shown in Fig. 2, when compared with the healthy persons, the population of CD3+ and CD4+ T lymphocytes was significantly decreased in HCC patients ($P < 0.05$), while the percentage of CD8+ T lymphocyte was markedly increased. The ratio of CD4+/CD8+ cells was also increased in HCC patients. Meanwhile, the proportion of NK cells in HCC patients was also significantly lower than that of the healthy persons ($P < 0.05$).

CD4+ T Cell Subsets are Increased Whereas CD8+ T Cell Subsets are Decreased in the AHCS Combined TACE-Treated HCC Patients

T cell population is an important indicator of human body's immune function. Recently, studies have demonstrated that T lymphocytes play an important regulatory role in the anti-tumor immune response, during which CD4+ cells promote effector cells to kill tumor cells. However, CD8+ cells are the main inhibitor of immune system. Therefore, we measured T lymphocyte population in peripheral blood in different treatment groups and the untreated group. As shown in Fig. 3a, when compared with the untreated group, the percentage of CD4+ T cells was increased in AHCS or TACE treatment group, but the difference was not significant. In contrast, CD4+ T cell subsets were dramatically increased in the AHCS combined TACE treatment group

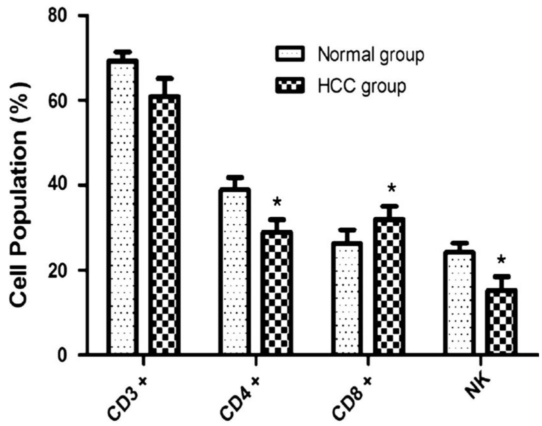

Fig. 2 The peripheral blood lymphocytes population in HCC patients and healthy persons before treatment. The difference was analyzed with two-sample *t* test. The *asterisks* indicate statistically significant difference ($P < 0.05$)

when compared with that in the untreated group ($P < 0.05$). On the contrary, the percentage of CD8+ T cell subsets was significantly decreased in AHCS and TACE treatment group ($P < 0.05$), and it further reduced in the AHCS combined TACE treatment group ($P < 0.01$, Fig. 3b). The ratio of CD4+/CD8+ T cells was also significantly increased in the AHCS combined TACE treatment group ($P < 0.01$, Fig. 3c).

NK Cells are Significantly Increased in the AHCS Combined TACE-Treated HCC Patients

NK cells are involved in the killing of tumor. They are able to directly dissolve and destroy tumor cells without pre-stimulation. We next measured NK cells in peripheral blood in different treatment groups and the untreated group. As shown in Fig. 4, when compared with the untreated group, the percentage of NK cells was increased in the AHCS and TACE treatment groups ($P < 0.05$), and they were markedly increased in the AHCS combined TACE treatment group when compared with that in the untreated group ($P < 0.01$).

Major Adverse Reactions in Each Treatment Group

127 liver cancer patients in this study successfully completed cryoablation catheter ablation and TACE surgery without intraoperative deaths. The major adverse reactions include fever, pain, discomfort, mildly elevated transaminase, bilirubin, and pleural effusions. The body temperature was mostly between 37.5 and 38.5 °C in TACE. Liver pain and discomfort is more common in the AHCS group, which gradually reduced after 3 days. Some patients in each treatment group had mild transaminase elevation, and

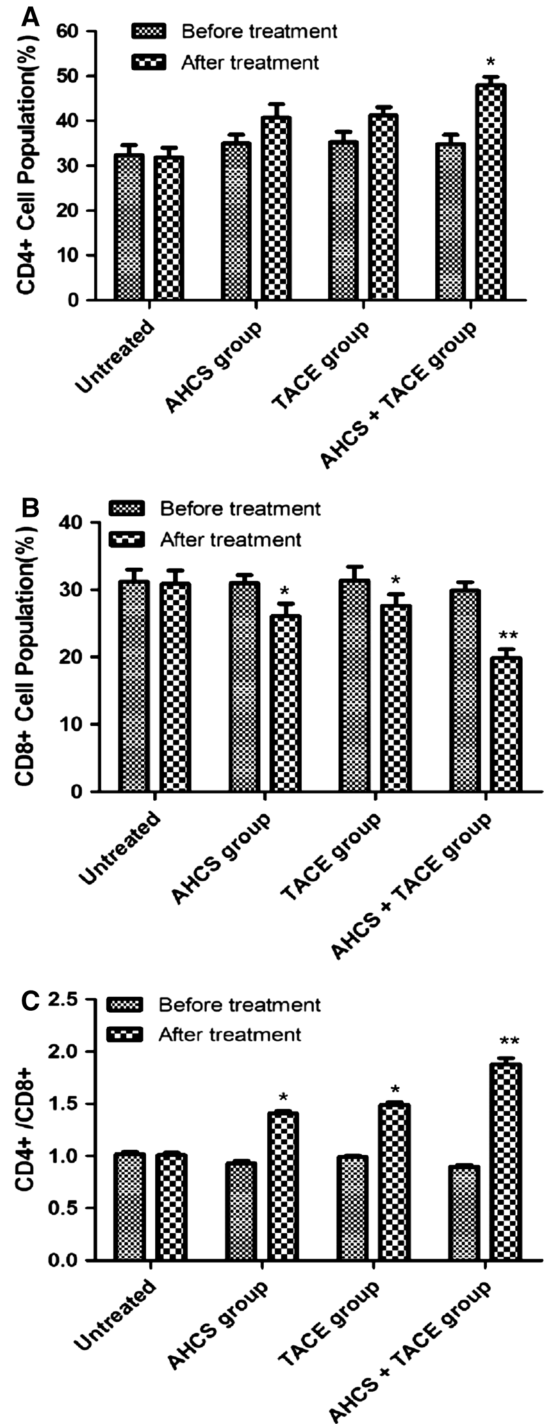

Fig. 3 T cell subsets in peripheral blood of HCC patients before and after treatment. **a** CD4+ cell population. **b** CD8+ cell population. **c** The ratio of CD4+/CD8+ cell subsets. The *asterisks* indicate statistically significant difference

a few patients had mildly elevated bilirubin. There was no significant difference in the liver function between the AHCS combined TACE treatment group, the AHCS group, and the TACE group. These results suggest that the AHCS

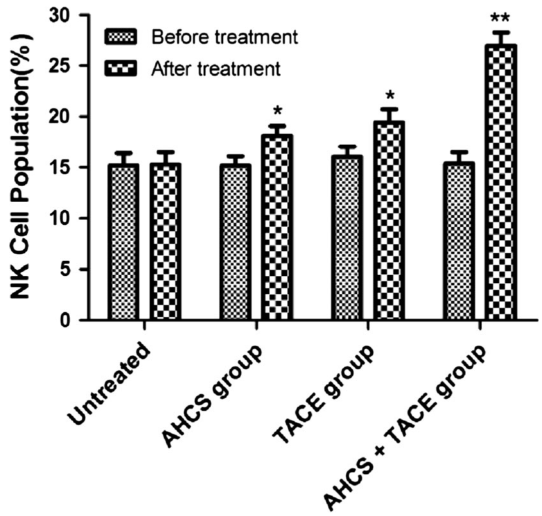

Fig. 4 NK cell population in HCC patients before and after treatment. The *asterisks* indicate statistically significant difference

combined with TACE treatment does not increase liver function damage. In addition, a few patients in the AHCS combined TACE treatment group have pleural effusion on their right side, which was improved after symptomatic treatment. The data are summarized in Table 1.

The Survival Rate at 6 months and 1 year was Significantly Increased in the AHCS Combined TACE-Treated Patients

The 6-month and 1-year survival rates are often used to evaluate the survival of HCC patients. Therefore, we further analyzed the effects of each treatment group based on the survival rate. As shown in Table 2, when compared with the untreated group, the 6-month survival rate increased in the treatment groups. The 1-year survival rate was significantly higher in the AHCS combined TACE treatment group when compared with that in the AHCS treatment group and the TACE alone group. Between the AHCS group and the TACE group, there was no statistically significant difference in the 6-month and 1-year survival rates.

Discussion

HCC treatment is difficult since the majority of patients are identified at late stage. For incompletely excised HCC, the preferred treatment is TACE. Through embolization of tumor blood vessels and chemotherapy drugs, TACE can remove most of the HCC cells and reduce tumor; however, TACE cannot cure liver cancer completely [16].

Recently, AHCS has been used for tumor treatment. There are several advantages of AHCS [17, 18]. AHCS is a minimally invasive operation system, which does not damage blood vessel [19]. It has good hemostatic effect that does not cause abdominal visceral organ bleeding [20]. In our cases, there was no obvious hemorrhage. Since freezing has an analgesic effect, AHCS may not cause pain in patients and therefore is easily accepted by patients. AHCS can cause immune response through freezing [21]. Tissues which were disintegrated by frozen ice hockey may increase the immune response, thereby inhibiting the residual cancer cells. AHCS can also be combined with other treatment methods such as TACE and percutaneous ethanol injection [22].

In this study, we combined AHCS with TACE to treat HCC. By measuring the index of AFP, T cell subsets, and NK cells, we show that AHCS, TACE, and combination of AHCS with TACE had significant treatment effects ($P < 0.05$). The combination treatment had better effect than AHCS or TACE treatment alone ($P < 0.01$). After 1 month of treatment, tumor volume was reduced and immunity was enhanced, which indicate that AHCS is able to enhance immune system function and activate anti-tumor immune response. Our data also suggest that treatment of HCC by combination of AHCS with TACE not only inhibits cancer cells more efficiently, but also activates anti-tumor immune system to fight tumor. We therefore suggest that the combination of AHCS with TACE can be a comprehensive therapeutic method for HCC. TACE attacks tumor cells with high concentration of chemotherapeutics. It also blocks blood supply for tumor growth. AHCS can induce tumor cell necrosis and activate innate immunity. In addition, AHCS removes tumor margin after TACE treatment and induces residual tumor cells necrosis without affecting the liver function [23]. Our data also show that

Table 1 Major adverse reactions in each group

Group	Cases	Pain	Fever	Pleural effusion	Hematologic	Transaminase	Bilirubin
Untreated	29	15	29	9	26	24	18
AHCS group	31	12	30	2	13	29	13
TACE group	32	12	31	0	12	31	14
AHCS + TACE group	35	13	33	3	14	32	12

Table 2 The survival rate at 6 months and 1 year in each group

Group	6-month survival rate (%)	1-Year survival rate (%)
Untreated (29 cases)	49.0	8.92
AHCS group (31 cases)	82.1	50.4
TACE group (32 cases)	88.5	55.3
AHCS + TACE group (35 cases)	90.8	79.8

combination of AHCS with TACE treatment for primary HCC is better than AHCS or TACE treatment when alone.

There is no doubt that better therapeutic effect can be achieved through the combined TACE and AHCS treatment, but it is still unclear if AHCS should be used before or after TACE, and the interval between the two methods. Studies have shown that hockey can induce faster, bigger, and more obvious frozen necrosis while interrupting hepatic blood [23]. Membrane permeability of cancer cell increased, which makes chemotherapy drugs more easily to get into cells after freezing. Moreover, TACE can also control small intrahepatic metastasis and keep the tumor easily frozen [24], reduce large ice hockey complications, such as rupture of liver and cold shock. All these data suggest that performing TACE treatment before AHCS might be a better strategy for HCC treatment.

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