·Original Article·

Serum alpha-fetoprotein measurement in predicting clinical outcome related to autologous cytokine-induced killer cells in patients with hepatocellular carcinoma undergone minimally invasive therapy

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[Abstract] Background and Objective: In patients with hepatocellular carcinoma (HCC) receiving potentially curative minimally invasive therapy, autologous cytokine-induced killer (CIK) cells were used to reduce recurrence. In this study we observed the changes in serum alpha-fetoprotein (AFP) after the treatment with CIK cells to explore if AFP could serve as a marker for predicting immunotherapeutic clinical outcome. **Methods:** A total of 122 patients with HCC and elevated AFP (> 25 ng/mL) received a curative treatment of transcatheter arterial chemoembolization (TACE) plus radiofrequency ablation (RFA) at the Sun Yat-sen University Cancer Center. Of these patients, 83 patients without residual tumor or extrahepatic metastasis and with AFP level less than 1.5 times the normal range (AFP < 37.5 ng/mL) were randomly assigned to the study group (n = 42) and the control group (n = 41). In the study group, CIK cells were transfused intravenously or via common hepatic arteries every week for at least 4 times, and the T-lymphocyte subset data before and after CIK cell infusions was examined by flow cytometry. All the two groups of patients were screened by tomography every 2 months to observe tumor recurrence. Serum AFP was collected at baseline and at different time points after treatment in parallel with radiologic response and clinical outcome. **Results:** Two patients in the control group were lost to follow-up after treatment. After CIK cell infusions, the downtrend of the AFP level was observed in the study group and not in the control group. There was a significant difference in the level of AFP between different time points after CIK infusions in both groups. The 1-year recurrence rate was 7.14 % for the study group and 23.1% for the control group (P = 0.044). In subgroup analysis, for patients with a slightly high level of AFP (25 ng/mL < AFP < 37.5 ng/mL) after curative TACE plus RFA treatment, the 1-year recurrence rate was 28.57% for the study group and 80% for the control group. The time to recurrence in the study group was also longer than that in the control group (mean 10.2 months vs. 6.8 months). After CIK cell infusions, the percent of $CD3^+CD4^+$ T cells and $CD4^+/CD8^+$ T cells increased from 28.1 ± 5.9% and 0.9 ± 0.3% to 32.7 ± 3.6% and 1.2 ± 0.2% (P < 0.001 and = 0.004, respectively), while the percent of $CD3^+CD8^+$ T cells decreased from 32.9 ± 8.4% to 28.8 ± 2.2% (P = 0.046). Also the percentage of patients with hepatitis B virus (HBV)-DNA content less than 1×10^3 copies/mL was 73.5% in the study group and 9.1% in the control group. **Conclusions:** CIK cells transfusion may reduce the level of serum AFP and anti-HBV and decrease the 1-year recurrence rate of patients with HCC after curative TACE plus RFA. Serum AFP decrease after CIK cell treatment may serve as a useful marker for predicting immunotherapy clinical outcome in patients with HCC undergone curative minimally invasive therapy.

Key words: Liver neoplasm, cytokine-induced killer cells, alpha-fetoprotein

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With general check-ups and routine health examinations, more patients with primary hepatocellular carcinoma (HCC) who carry the hepatitis B virus (HBV) are diagnosed early. Previously comprehensive treatment focused on surgery, while in recent years, minimally invasive therapy for patients with HCC has been fully confirmed by evidence-based medicine (EBM). The

treatment pattern for patients with HCC has gradually evolved from previous surgical treatment alone to the current multidisciplinary specialization minimally invasive comprehensive treatment [1,2]. Transcatheter arterial chemoembolization (TACE) combined with image-guided radiofrequency ablation (RFA) is one of the comprehensive strategies and has obtained good efficacy in treating patients with HCC [3]. However, due to combined with hepatitis and sclerosis in HCC patients and the biologic characteristics of multicenter origins occurs synchronously or heterochronously [4,5], as well as the disorders of immune functions in patients with tumors [6], recurrent rates are higher even with curative treatment. The efficacy of the curative treatment is thus compromised. Therefore, the investigation for an effective method to strengthen treatment efficacy and to prevent and inhibit the recurrence of HCC is a hot issue in the research of HCC.

Studies have suggested that the factors and potential mechanisms may vary for recurrence developed at different time points after treatment for HCC. Long-term recurrence may be due to new lesions originating from varying HCC clones, but short-term recurrence is probably due to uncontrolled local tumors and micrometastatic lesions derived from tumor clones. Therefore, an important step in preventing recurrence is to improve local control and eradicate micrometastatic lesions [7]. Minimally invasive therapy is particularly useful in terms of local control, while cell immune therapy exerts a wide range of antitumor activities by mechanisms of action that include improving immune functions in the body and directly eradicating residual tumor cells in the patients. Cytokine-induced killer (CIK) cells are one of these immune therapies and present potent proliferation in vivo as well as strong antitumor activity. Clinical studies have demonstrated that it is an excellent method to prevent tumor recurrence and has preliminarily shown its efficacy in inhibiting recurrence and metastasis of primary HCC [8,9].

Moreover, among numerous factors that predict HCC recurrence, alpha-fetoprotein (AFP) has been proved by a body of EBM evidence to be in close correlation with recurrence [7,10]. AFP is a protein that can be expressed by hepatic cancer cells, with extremely complicated biologic activities. Studies have shown that AFP plays double roles in both inhibiting the immune system and promoting the growth of cancer cells. For the time being, it is still unclear whether such roles have an effect in HCC recurrence. Although 10%–30% of patients with HCC have negative AFP expression, most investigators believe that AFP is significant in predicting HCC recurrence and evaluating the biologic features of tumors and treatment regimens. Recent studies have gradually established the role of AFP as a tool in evaluating efficacy of systemic chemotherapy for patients with advanced HCC [11,12], but no studies have been reported regarding its role as a tool for evaluating the efficacy as a biologic therapy.

Herein we investigated the efficacy of adoptive cell immunotherapy combined with curative minimally invasive therapies in preventing HCC recurrence after minimally invasive therapy and monitored the changes in serum AFP values during CIK cell therapy in a randomized controlled study, which revealed that CIK cells eradicated residual cancer cells and prompted a decrease of AFP value from the upper limit of the normal range to the lower limit of normal after CIK cells therapy, to investigate the significance of AFP as a marker in evaluating clinical efficacy of CIK cells.

Materials and Methods

Study subjects and inclusion criteria

Between July 2002 and August 2008, 122 selected patients with HCC were treated using TACE with combined sequential RFA therapy at the Center of Medical Radiology and Intervention in Sun Yat-sen University Cancer Center.

Inclusion criteria included (1) all patients provided informed consent for the therapies before inclusion; (2) diagnostic criteria for HCC: the patient fulfilled the Clinical Diagnostic Criteria for HCC in 2001 and was ratable by the clinical staging system. For patients diagnosed after 2005, the American Association for the Study of Liver Disease (AASLD) criteria was adopted [13]; (3) all patients had their liver function parameters within normal ranges and had an AFP value higher than the normal reference value (25 ng/mL) when first diagnosed; (4) computed tomography (CT) scanning suggested major intrahepatic tumors were mass-shaped. For those with multiple cancer nodules, the number of nodules was should be no more than 3; (5) radiology suggested no cancer embolus formation in the inferior vena cava or the portal vein, and no distant metastasis was identified; (6) at 6 to 8 weeks after TACE and sequential RFA therapy, the possibility of residual tumors was excluded by contrast-enhanced CT scanning, enhanced magnetic resonance imaging (MRI) examination, or positron emission tomography (PET)/CT together with the results of clinical examinations. In addition, AFP level fell back to normal range or remained higher than the reference value but lower than 1.5 times of the upper limit of the reference value (that is, < 37.5 ng/mL), however digital subtraction angiography (DSA) suggested no intrahepatic tumor blush; and (7) before randomization, two consecutive AFP determinations, with at least a 1-week interval between them, suggested that the AFP concentration was stable (variation of less than 50%).

Apparatus and main reagents

TACE was performed with DSA (Toshiba Corporation, BLA-800A). The CT scanning system used was a Marconi CT-twin flash at the scanning conditions: 120 kV; 265 mAs; slice thickness: 8–10 mm; and pitch: 1. RFA systems were WE7568 multipolar radiofrequency ablation systems for tumors at the pulse power of 400 W and the pulse frequency of 290 kHz, or a monopolar cool-tip therapy system (Cool-Tip; Valleylab, MA, USA). For selective TACE therapy, the routine mixture emulsion of pirarubicin (50–60 mg), mitomycin (8–10 mg), cisplatin (20–60 mg), oil solution and iodized oil (10–20 mL) was used (in each specific case, the dose of the drugs mentioned above might be modified as appropriate according to tumor size and tumor blood supply).

Preparation and infusion of CIK cells

After the RFA procedure for HCC, CIK cells were prepared in accordance with the routine operations in our biologic therapy center. When final preparation of the CIK cells fulfilled the criteria of acceptability at our center[9], CIK cells infusion was performed once every week, with a total of at least 4 infusions and more than 1×10^{10} cells for each infusion. Infusion was given via common hepatic artery or peripheral veins.

Supplemental clarification before inclusion

After curative TACE combined with RFA therapy, routine liver protection and supportive treatments were given, and patients were randomly assigned and included for CIK cell therapy when liver functions were within normal ranges.

Detections of AFP and T-lymphocyte subsets in peripheral blood

Patients underwent routine AFP examinations after being admitted, at 1 week and 4 weeks after TACE and sequential RFA procedures. In the study group, AFP detection was performed before each CIK cell therapy and once every 4 weeks within 1 year after the end of CIK cell therapy. For the control group, AFP detection was performed once every 4 weeks within 1 year after minimally invasive therapy. After 1-year follow-up, AFP detection was performed once every 2 months for all patients.

In the study group, 2 mL blood was obtained in peripheral blood before CIK cell therapy and after at least 2 CIK cell infusions for routine detection of lymphocyte subsets.

Follow-up and evaluation of efficacy

Patients were followed up for at least 1 year, during which the tumor was evaluated once every 1–2 months. After the first year, the tumor was evaluated to once every 2–3 months with follow-up contrast-enhanced liver CT scanning (those who were allergic to contrast media could be given enhanced MRI scanning) and routine biochemical tests, HBV-DNA content, chest X-ray together with the results of AFP detections, to determine whether recurrence developed. When the determination was not possible, ultrasonography and MRI would be used in combination, and local biopsy could be performed when necessary.

Statistical methods

AFP data obtained before and after treatment and during follow-up were presented as mean ± standard deviation (SD) and were described and analyzed by the SPSS version 15.0, with the study design of a *t* test for self-paired means. The 1-year recurrent rates in the study and control groups were analyzed with a χ^2 test. A *P* value of less than 0.05 was considered as statistically significant.

Results

A total of 83 patients met inclusion criteria and were randomly assigned using sealed envelopes into two groups: the study group (n = 42) and the control group (n = 41). Of the 83 patients, 81 completed the follow-up and 2 patients in the control group were lost to follow-up after the procedures. The characteristics of 81 patients with HCC were well balanced between the two groups (Table 1).

Table 1 Characteristics of 81 patients with HCC randomly assigned to a study group and a control group after TACE plus RFA

Characteristic	Study group (n=42) (CIK + TACE + RFA)	Control group (n=39) (TACE + RFA)	*P*
Age (years)			> 0.05
Range	35–78	38–70	
Mean	53.0 ± 11.7	54.5 ± 11.0	
Gender			> 0.05
Male	37	34	
Female	5	5	
Serum AFP level[a]			> 0.05
25–100 ng/mL	4	3	
101–400 ng/mL	5	5	
> 400 ng/mL	33	31	
Tumor diameter			> 0.05
⩽ 5 cm	32	30	
> 5 cm	10	9	
Tumor number			> 0.05
Single	35	33	
Multiple	7	6	> 0.05
HBsAg positive	39	36	
HBV-DNA			> 0.05
103–106 copies/mL	14	9	
> 106 copies/mL	1	2	
Baseline AFP[b]			> 0.05
< 25 ng/mL	32	30	
25–37.5 ng/mL	10	9	

HCC, hepatocellular carcinoma; TACE, transcatheter arterial chemoembolization; RFA, radiofrequency ablation; CIK, cytokine-induced killer cells; AFP, alpha-fetoprotein.
[a] Before minimally invasive therapy; [b] at the time of randomization.

Outcome of CIK cell immunotherapy in the study group

A total of 42 patients in the study group underwent CIK cell immunotherapy, of whom 12 patients received CIK cells via common hepatic artery and 30 patients received CIK cells via peripheral veins. In addition, 10 patients received 8 infusions and 4 patients received 12 infusions.

Changes in AFP concentration

In the control group, no downtrend of AFP concertration was observed when comparing AFP concentrations during follow-up after TACE and the combined RFA procedure to baseline levels at inclusion (Figure 1). In the study group where minimally invasive therapy was combined with CIK therapy, AFP concentrations during follow-up gradually decreased when comparing to baseline levels before inclusion, and AFP levels were maintained when it was reduced to lower concentrations (Figure 2). The differences in AFP concentrations at various

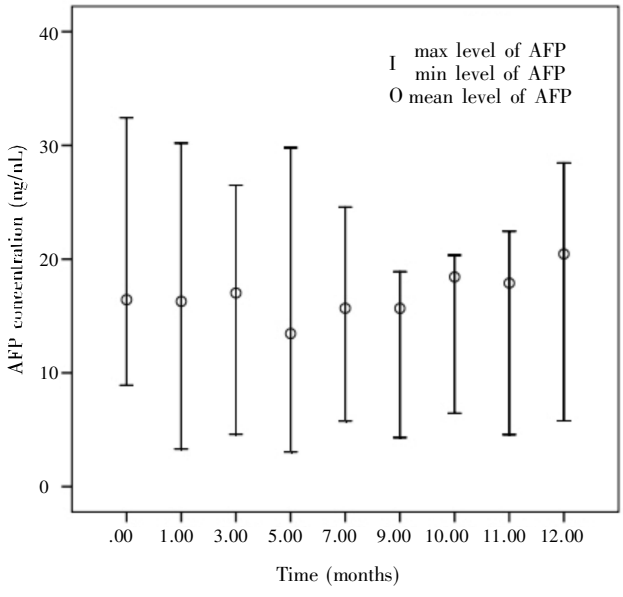

Figure 1 Change of AFP concentration in control group

No downtrend of AFP concentration was observed in the control group when comparing AFP concentration during follow-up to baseline levels at randomization.

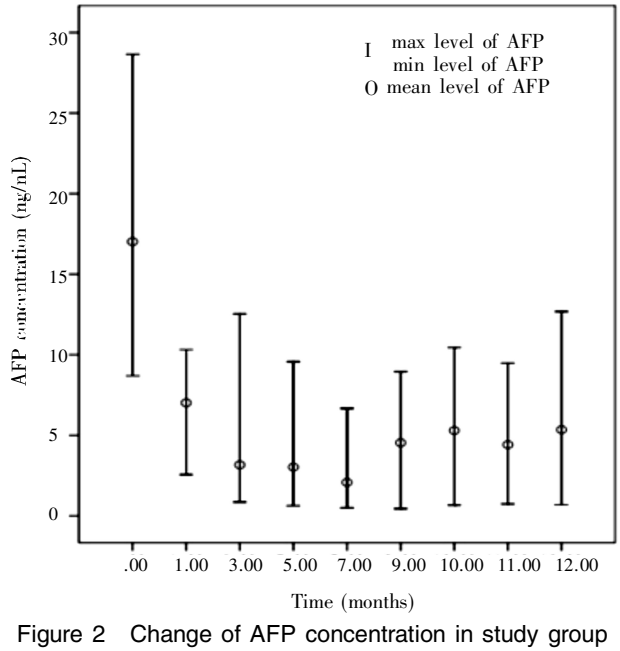

Figure 2 Change of AFP concentration in study group

After the trentment with CIK cells, the downtrend of AFP concentration was observed in study group compared with baseline levels at randomization.

follow-up time points were significant between the two groups (Table 2).

Changes in T lymphocyte subsets of peripheral blood and HBV-DNA content

In the study group, results of detections of T lymphocyte subsets in peripheral blood before and after CIK cell therapy were as follows. The ratio of $CD3^+CD4^+$ T cells significantly increased, while $CD3^+CD8^+$ T cells significantly decreased, and thus the $CD4^+/CD8^+$ T cells ratio significantly increased. These values evolved from 28.1 ± 5.9, 32.9 ± 8.4, and 0.93 ± 0.32, respectively, before therapy to 32.7 ± 3.6, 28.8 ± 2.2, and 1.16 ± 0.19, respectively, after therapy (P values were < 0.001, = 0.046, and = 0.004, respectively).

In the study group, with combined CIK cell therapy, the HBV-DNA content was higher than 1.0×10^3 copies/mL in 15 patients, while only in 4 patients after therapy. Among these, the HBV-DNA content was within the range of 1.0×10^3–1.0×10^6 copies/mL in 14 patients, while only in 3 patients after therapy. In one patient whose HBV-DNA content was more than 1.0×10^6 copies/mL before therapy, the value was 1.6×10^4 copies/mL after therapy. As for the control group, the HBV-DNA content before therapy was higher than 1.0×10^3 copies/mL in 11 patients. During the 1-year follow-up, the HBV-DNA content was reduced from 1.1×10^5 copies/mL before therapy to less than 1.0×10^3 copies /mL after therapy in only one patient.

Comparisons of tumor recurrence

All patients were followed up for more than 1 year. In the control group, 9 patients developed recurrence within 1 year, of whom 6 patients had an AFP concentration slightly higher than the reference value after the procedures. The mean AFP concentration was 28.03 ± 6.44 ng/mL, and time to recurrence was 4.3 months, 5.4 months, 6.2 months, 7.0 months, 7.6 months, and 10.3 months, respectively. In the study group, 3 patients had recurrence within the 1-year follow-up, of whom 2 patients had AFP concentration slightly higher than the reference value after the procedures. After CIK cell immunotherapy, AFP concentrations reduced in all patients, but mean AFP value was more than 5 ng/mL. Time to recurrence was 9.4 months and 11.2 months, respectively, after CIK cell therapy.

The 1-year recurrence rate was 23.1% (9/39) for the study group and 7.14% (3/42) for the control group (P = 0.04). After curative TACE and the combined RFA procedure, the recurrence rate in patients with AFP concentration slightly higher than the reference value significantly decreased in the adjuvant CIK cell therapy group (the study group) as compared to the control group (20% vs. 66.67%), and time to recurrence was shortened

Table 2 Change of AFP concentration in the study group and the control group after TACE plus RFA

Group	Baseline	1 month after treatment	3 months after treatment
Study (n=42)	17.02 ± 11.96	7.02 ± 3.58	3.17 ± 1.16
Control (n=39)	16.43 ± 12.89	16.28 ± 10.89	17.02 ± 11.38
P	> 0.05	< 0.05	< 0.05

as compared to the control group as well (average 6.8 months vs. 10.2 months).

Discussion

AFP is currently widely recognized as a tumor-related antigen for HCC, with extremely complicated biologic activities. While its exact biologic function is still unclear, it may be related to tumor occurrence and development. In adults its serum level is fairly low, approximately 5–10 ng/mL, but cancerous hepatic cells can synthesize AFP[14]. Theoretically, since the half-life of AFP is less than 5 days, AFP levels may decrease to normal levels at approximately 30 days (about 5–6 half-lives) after effective curative treatment in AFP-positive HCC[15]. But concomitant infection with HBV and impairment from hepatic sclerosis and in the hepatic parenchyma are also factors that lead to increase of AFP levels[16-18]. In addition, it is also possible that there may be micrometastatic lesions or residual cancer cells derived from HCC colony in blood circulation, therefore AFP expression change to negative in only 80.3% of patients even after surgery[19]. In this study, AFP expression change to negative in 76.5% (62/81) of the patients at 6–8 weeks after curative minimally invasive therapy in a group of patients with AFP positive HCC, which was consistent with the reported literature[19]. In our study, the reasons why AFP was slightly higher than normal after curative minimally invasive therapy in some patients might include the mechanisms mentioned above. These factors were well balanced in the distribution between the two groups of patients. While after CIK cell therapy, serum AFP level in the patients decreased further, the pathway by which AFP decreased might therefore be related to the CIK cell therapy. The results of our study primarily demonstrated the effect of CIK cell therapy in reducing short-term (1-year) recurrence after minimally invasive therapy for HCC. After curative minimally invasive therapy for HCC, the recurrence rate within 1 year in patients with an AFP level slightly higher than the reference value after the procedures was lower in the CIK cell therapy group (the study group) than in the control group, and time to recurrence was prolonged as well. These indicate that further and continued decreases in AFP concentrations after CIK cell therapy might be the pathway via which CIK cells exert their roles in preventing short-term recurrence, and that further and continued decreases in AFP concentrations might be a candidate marker to predict the clinical efficacy of CIK cells as adjuvant therapy for minimally invasive therapy for patients with HCC.

Studies have suggested that AFP expressed by hepatic cancer cells are endowed with the activity of inducing apoptosis in lymphocytes and inhibiting immune functions[20], while immune hypofunction is an important reason for the susceptibility to HCC recurrence and metastasis. AFP may inhibit immune function in the body by altering the ratio of $CD4^+$ and $CD8^+$ T lymphocyte subsets and inducing lymphocyte death[21,22]. The results of our study revealed that serum AFP concentration reduced in the patients after CIK cell infusion, and among the peripheral T lymphocyte subsets, the ratio of $CD3^+CD4^+$ T cells significantly increased, $CD3^+CD8^+$ T cells significantly decreased, and thus the ratio of $CD4^+/CD8^+$ increased, which was consistent with the results from previous studies[9]. That is, the changes in T lymphocyte subsets reflected improvement at various degrees in immune function of the body, and immune suppression on the body by tumor cells was attenuated and antitumor capacity was enhanced. These effects may be very important in inhibiting tumor recurrence and metastasis[2]. We deduced that the improvement in immune function resulting from the CIK cell therapy might be partly due to the decrease in AFP. Such change prevented AFP from inhibiting immune functions by altering the ratio of $CD4^+$ and $CD8^+$ T lymphocyte subsets.

AFP expressed by hepatic cancer cells may also be an important endogenous autocrine protein that promotes tumor cell proliferation[23]. Such activity in promoting cancer cell growth is not only due to the immune suppression of AFP, but also because AFP accelerates the transformation from G_0-phase cells to S-phase cells and significantly prompts DNA synthesis. The expression of oncogenes, such as p53, c-fos, c-jun, and N-ras, also increases in AFP-treated cells[24]. Hence, the mechanism via which CIK cells reduced and delayed tumor recurrence in the study group might be that CIK cell therapy could reduce serum AFP concentrations and interrupt the promotion of AFP on cancer cell growth. Even though there were small amounts of residual tumor cells in the body, the absence of adequate promotion on cancer cell growth delayed tumor recurrence and metastasis.

Moreover, studies have suggested that AFP may induce hepatic cancer cells to escape from immune surveillance in the body. Its mechanisms may be related to AFP helping hepatic cancer cells escape from immune surveillance by numerous mechanisms, including influencing the expression of the Fas/FasL system in hepatic cancer cells via P53 and regulating the expression of tumor necrosis factor (TNF) receptors[25,26]. Therefore, decreases in AFP concentrations may prevent hepatic cancer cells from escaping from immune surveillance, and thus allow endogenous immunity and/or exogenous immunotherapy to fully exert their effects in eradicating residual tumor cells and reducing or preventing the recurrence and metastasis given rise by immune-tolerant cells. Such an AFP-related mechanism may be another mechanism via which CIK cell therapy delays tumor recurrence and metastasis.

Previous studies have demonstrated that adoptive cell immunotherapy in patients after surgical resection for HCC could reduce the recurrent rate of HCC, and its mechanisms may be related to CIK cell therapy directly eradicating cancer microlesions, counteracting HBV, and improving immune function[8]. In this study, it was observed that CIK cell therapy presented good efficacy in reducing short-term tumor recurrence.

Overall, its mechanisms may be (1) CIK cell therapy directly eradicates residual intrahepatic cancer microlesions after TACE combined with RFA, effectively eliminating residual tumor cells, and thereby improving the efficacy of comprehensive treatment for primary HCC; (2) CIK cell therapy reduces the content of HBV in the body; CIK cells may have a role in counteracting HBV and prevents HBV from replicating, therefore prevents the recurrence of HCC induced by the pathway of HBV replication, which is consistent with other reports[27]; and (3) our study found that AFP concentration in patients with HCC significantly reduced after CIK cell infusion. Possible mechanisms might be that CIK cell therapy eradicated residual microtumor cells that secreted AFP and thus decreased the AFP level. It might have improved the suppression of immune function in the body from AFP by interrupting the pathway via which AFP promoted proliferation of HCC cells[21], and thereby achieved its effect in inhibiting tumor recurrence and metastasis.

Our study observed a limited number of patients, but primarily revealed the phenomenon and tendency that CIK cell therapy could further decrease AFP levels after TACE and sequential RFA in patients with AFP-positive HCC. By detecting the changes in the ratios of T lymphocyte subsets, our study also elaborated that the mechanism of CIK reducing tumor recurrence might include AFP pathways. In addition, our study observed the short-term clinical recurrent rates in the two groups of patients and the comparison primarily showed that a sustainable decrease of AFP to a lower level might be a predictor marker for excellent clinical efficacy of CIK cells. Our study also raised rethinking of the decrease of AFP to normal levels after treatment in patients with AFP-positive HCC. That is, AFP values at the upper limits of the normal range might not indicate complete eradication of residual cancer cells in patients, while dynamic and continued decrease of AFP might suggest elimination of residual cancer cells and predict favorable short-term prognosis for patients. Dynamic monitoring of AFP values has important clinical implications and should be widely used in clinical settings. Further correlation between CIK cell therapy and AFP and its clinical implications have yet to be further investigated.

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