Hepatocellular carcinoma (HCC) is the fifth most common cancer in the world, with more than 80% of the cases occurred in Asia. HCC become the second most common cause of cancer death in China. Treatment of HCC remains a critical issue, particularly in China, as it is estimated that Chinese patients account for 40% of HCC cases worldwide. Surgical resection is the main curative treatment. Unfortunately, only around 20% of HCC patients may benefit from surgical therapy. Most patients are diagnosed too late, already presenting with advanced disease. Transcatheter arterial chemoembolization (TACE), which has shown a survival benefit, is now widely adopted for unresectable HCC. However, the recurrence rate after TACE is still high and the long-term survival is unsatisfactory. It is a challenge to enhance the efficacy of TACE and reduce recurrence after TACE. It is well known that improving the overall therapeutic effects on HCC depends on the combined therapies.

Cytokine-induced killer (CIK) cells are the major histocompatibility complex-unrestricted cytotoxic lymphocytes and generated by incubating peripheral blood monocytes (PBMC) with various types of cytokines such as CD3 monoclonal antibody, interleukin-2 (IL-2), IL-1, and interferon-gamma (IFN-γ). The high anti-tumor activity of CIK cells is mainly due to the high proliferation of CD3⁺CD56⁺ cells. Some reports indicated that CIK cell therapy can be used as an efficient adjuvant anticancer immunotherapy to eradicate residual cancer cells and prevent or postpone tumor relapse. In the present nonrandomized study, we compared therapeutic efficacy of TACE alone or in combination with CIK cell therapy on HCC in terms of progression-free survival (PFS) and overall survival (OS).

Materials and Methods

Selection of patients
From May 2005 to September 2008, 146 HCC patients over
18 years old were enrolled according to inclusion and exclusion criteria (Table 1). The diagnosing criteria of HCC was made according to the Diagnosing and Staging National Standards of China (2001) for hepatocellular carcinoma[5]. According to the wishes of patients, they were divided into combination group (72 patients were treated with CIK cell therapy combined with TACE) and TACE group (74 patients were treated only with TACE). No significant differences in baseline demographics were noted between the two groups (Table 2).

### Table 1 Criteria for Inclusion and Exclusion of HCC patients

<table>
<thead>
<tr>
<th>Inclusion criteria</th>
<th>Exclusion criteria</th>
</tr>
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<tbody>
<tr>
<td>Men and women &gt;18 years of age</td>
<td>Infiltrative or diffuse HCC</td>
</tr>
<tr>
<td>HCC diagnosed by high level of serum AFP (≥ 400 ng/mL) with typical imaging findings, or confirmed by needle liver biopsy while AFP &lt; 400 ng/mL</td>
<td>Significant cardiovascular disease such as myocardial infarction occurred within recent 6 months, chronic heart failure or unstable coronary artery disease</td>
</tr>
<tr>
<td>Patients had unresectable HCC or refused resection</td>
<td>Systemic chemotherapy or angiogenesis inhibitor therapy before disease progression</td>
</tr>
<tr>
<td>Total bilirubin &lt; 3 × upper limit of normal</td>
<td>Patients with other malignant tumor within the past 5 years before treatment</td>
</tr>
<tr>
<td>Child-Pugh stage A or B</td>
<td>Pregnant or breastfeeding patients</td>
</tr>
<tr>
<td>No extrahepatic metastasis</td>
<td>Patients with uncontrolled infections or HIV-seropositive patients</td>
</tr>
<tr>
<td>INR/PTT &lt; 1.5 × upper limit of normal</td>
<td>History of organ transplantation</td>
</tr>
<tr>
<td>Written informed consent</td>
<td>Patients with hemorrhage/bleeding event</td>
</tr>
<tr>
<td>Newly diagnosed or had postoperative recurrence</td>
<td>Mental conditions rendering the patient incapable to understand the nature, scope, and consequences of the study</td>
</tr>
</tbody>
</table>

HCC, hepatocellular carcinoma; AFP, alpha-fetoprotein; HIV, human immunodeficiency virus; INR/PTT, international normalized ratio/prothrombin time.

### Table 2 Baseline characteristics of the patients

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Combination group</th>
<th>TACE group</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total cases</td>
<td>72</td>
<td>74</td>
</tr>
<tr>
<td>Median age (years)</td>
<td>53</td>
<td>51</td>
</tr>
<tr>
<td>Gender</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>65</td>
<td>64</td>
</tr>
<tr>
<td>Female</td>
<td>7</td>
<td>10</td>
</tr>
<tr>
<td>Serum hepatitis B surface antigen</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Positive</td>
<td>68</td>
<td>68</td>
</tr>
<tr>
<td>Negative</td>
<td>4</td>
<td>6</td>
</tr>
<tr>
<td>Serum AFP</td>
<td></td>
<td></td>
</tr>
<tr>
<td>≤ 20 ng/mL</td>
<td>20</td>
<td>17</td>
</tr>
<tr>
<td>21–399 ng/mL</td>
<td>17</td>
<td>12</td>
</tr>
<tr>
<td>≥ 400 ng/mL</td>
<td>35</td>
<td>45</td>
</tr>
<tr>
<td>Child-Pugh classification</td>
<td></td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>65</td>
<td>66</td>
</tr>
<tr>
<td>B</td>
<td>7</td>
<td>8</td>
</tr>
<tr>
<td>Portal vein thrombosis</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Positive</td>
<td>13</td>
<td>22</td>
</tr>
<tr>
<td>Negative</td>
<td>59</td>
<td>52</td>
</tr>
<tr>
<td>Arteriovenous fistula</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Positive</td>
<td>12</td>
<td>18</td>
</tr>
<tr>
<td>Negative</td>
<td>60</td>
<td>56</td>
</tr>
<tr>
<td>BCLC stage</td>
<td></td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>7</td>
<td>5</td>
</tr>
<tr>
<td>B</td>
<td>6</td>
<td>4</td>
</tr>
<tr>
<td>C</td>
<td>59</td>
<td>65</td>
</tr>
<tr>
<td>ECOG performance status</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0–1</td>
<td>66</td>
<td>61</td>
</tr>
<tr>
<td>2–3</td>
<td>6</td>
<td>13</td>
</tr>
<tr>
<td>Times of TACE</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>18</td>
<td>29</td>
</tr>
<tr>
<td>≥ 2</td>
<td>54</td>
<td>45</td>
</tr>
</tbody>
</table>

TACE, transcatheter arterial chemoembolization; AFP, alpha-fetoprotein; BCLC, Barcelona Clinic Liver Cancer Staging; ECOG, the Eastern Cooperative Oncology Group.

### Treatment procedure

The two groups received TACE according to a standard protocol. Patients fasted for 8 h before TACE. Intravenous injection of triptisepron (5 mg) was given before the procedure. The femoral artery was catheterized under local anesthesia. Hepatic arteriography and superior mesenteric arterial portovenography were performed to define the size and location of tumor nodules and to identify occlusion of the main portal vein. The right or left hepatic artery feeding the tumor was superselectively catheterized. By the pumping method, oxaliplatin was mixed with lipiodol in a ratio of 100 mg to 10 mL to make into an emulsion. Various amounts of the emulsion, up to a maximum of 40 mL of lipiodol (containing 200 mg of oxaliplatin) were injected slowly under fluoroscopic monitoring according to the size of the tumor and the arterial blood flow to deliver a sufficient amount of the emulsion to the tumor areas without retrograde flow. If the tumor involved both lobes of the liver, or if superselective catheterization was not possible, the emulsion was injected into the proper hepatic artery distal to the origin of the gastroduodenal artery. Fluorouridine (1 000 mg) or gemcitabine (1 600 mg) was injected into the common hepatic artery before lipiodol embolization. If possible, remnant oxaliplatin were injected into the common hepatic artery after lipiodol embolization, followed by embolization with small gelatin-sponge pellets of 1 mm in diameter. Chemoembolization was repeated in 30 to 45 days and was withheld or discontinued whenever vascular contraindications, poor hepatic function, severe adverse events, or progressive disease with a diffuse growth pattern developed.

CIK cells were isolated and cultured according to a standard protocol. Using a blood cell separator, (2–4) × 10⁶ PBMC cells from each patient were obtained in a total volume of 50–60 mL. Cells were resuspended in phosphate buffered saline (PBS).
without calcium and magnesium. Cell concentration was adjusted to $1.0 \times 10^5$/ml in RPMI-1640 medium. PBMC cells were incubated with 1000 U/ml rhIFN-γ for 24 h, then added with 50 ng/mL anti-CD3 mAb, 100 U/ml rhIL-10, and 500 U/ml rhIL-2. Fresh rhIL-2 and fresh RPMI-1640 medium were replenished every 3 days. CD3⁺CD56⁺, the major immunophenotype of CIK cells, was examined 10 days after incubation. when the cell number reached more than $1 \times 10^8$, CIK cells were collected within 24 h before TACE, then centrifuged at 1000 rpm to remove the medium, washed with normal saline (NS) for 3 times and resuspended in 100 mL NS, and transfused back into HCC patients in combination group via the vein on days 10, 13, 15, 18, respectively, according to protocol. Successive 4 times of transfusion was a course of treatment. The number of transfused CIK cells per patient was $1\sim5 \times 10^8$ in one course of treatment. A maximum of 4 courses of treatment was given in one patient every year. No patient accepted extra cytokine treatment.

Assessment of outcome

The primary end points were progression-free survival (PFS) and time-to-progression (TTP); the second end point was overall survival (OS). TTP was defined as the interval from the beginning of treatment to death or disease progression. The patients were followed monthly at the outpatient clinic till March 15, 2009. Serum biochemistry, serum alpha-fetoprotein (AFP) detection, and CT or MRI were repeated every month in the first trimester, then every two months. All patient deaths were the end point irrespective of the cause of death. TACE-related death was designated as death within 30 days after the initial therapy.

Statistical analysis

Intergroup comparison was made on an intention-to-treat basis. The frequency of each variable was analyzed by the Chi-square test and comparisons of group means were performed using the Student’s t test. Univariate analysis for baseline variables to identify predictors of survival was performed by estimating the survival rate according to the Kaplan-Meier method and compared using the log-rank test. The PFS and OS curves of the two groups were then compared with stratification according to significant prognostic factors. All the significant prognostic factors related to PFS and OS identified from univariate analysis were put into a Cox proportional hazards model for multivariate analysis. The level of significance was set at $P < 0.05$. Statistical analysis was performed with the SPSS13.0 software.

Results

Patient characteristics

All patients received a total of 279 courses of TACE (median, 2 courses; range, 1–4 courses) before disease progression, and the 72 patients of combination group received a total of 111 courses of CIK cell transfusion (median, 1 course; range, 1–3 courses) before disease progression. One patient in the TACE group were lost and could not be contacted after a follow-up of 32 months. At the time of the final analysis, 40 patients in TACE group and 28 patients in combination group had died. According to RECIST (Response Evaluation Criteria in Solid Tumors), the short-term responses of the two groups were similar. No patient reached complete remission (CR); 13 (18.0%) patients in combination group and 11 (14.9%) in TACE group reached partial remission (PR); 59 (81.9%) patients in combination group and 63 (85.1%) in TACE group had stable disease (SD).

Progression-free survival

The 6-month, 1-year, and 2-year PFS rates were 72.2%, 40.4%, 25.3% in combination group, and 34.8%, 7.7%, 2.6% in TACE group. The median TTP was 11 months [95% confidence interval (CI), 8–14 months] for combination group and 5 months (95% CI, 4–7 months) for TACE group (Figure 1). The PFS was significantly better in combination group than in TACE group ($P < 0.001$). The median PFS increased by 6 months (120% improvement), from 5 months (TACE group) to 11 months (combination group).

By univariate analysis, portal vein thrombosis, Child-Pugh classification, ECOG performance status, BCLC stage, CIK cell therapy, and times of TACE before disease progression were associated with PFS. Meanwhile, multivariate Cox proportional hazard analysis demonstrated that ECOG performance status, CIK cell therapy, and times of TACE before disease progression were the independent prognostic factors that affected PFS (Table 3).

Of the 74 patients in TACE group, 63 (85.1%) had disease progression, 11 (14.9%) had extrahepatic metastasis. Of the 72 patients in combination group, 51 (70.8%) had disease progression, 10 (13.9%) had extrahepatic metastasis. The difference in disease progression was not significant between the two groups ($P = 0.769$).

For the patients received one time of TACE before disease...
progression, there was no significant PFS difference between the two groups ($P = 0.133$); for those received 2–4 times of TACE, the PFS was significantly better in combination group than in TACE group ($P < 0.001$). For the patients with ECOG performance status of 0–1, the PFS was significantly better in combination group than in TACE group ($P < 0.001$); for those with ECOG performance status of 2–3, the difference was not significant between the two groups ($P = 0.450$).

**Overall survival**

The estimated 6-month, 1-year, and 2-year OS rates were 90.3%, 71.9%, 62.4% in combination group, and 74.6%, 42.8%, 18.8% in TACE group. The median OS was 31 months (95% CI, 26.7–35.3 months) for combination group and 10 months (95% CI, 7.3–12.7 months) for TACE group (Figure 2), with significant difference ($P < 0.001$).

Univariate analysis showed that portal vein thrombosis, Child-Pugh classification, ECOG performance status, BCLC stage, arteriovenous fistula, CIK cell therapy, and times of TACE were associated with OS. With multivariate analysis, the times of TACE, ECOG performance status, and CIK cell therapy were independent prognostic factors of all patients (Table 5).

**Discussion**

Our study has shown that adjuvant immunotherapy with CIK cells may greatly prolong PFS and OS of HCC patients after TACE.

HCC is a common malignant tumor in Asia. Hepatic resection offers a chance of cure for a minor proportion of patients with early stage tumor and preserved liver functions. Because of the shortage of organ donors, the role of liver transplantation in treatment remains limited. The majority of the patients with unresectable HCC are treated by various palliative therapies. TACE is the most widely used treatment for unresectable HCC with proven improvement on survival in selected patients with well preserved liver function. The goal of TACE is to deliver a high dose of chemotherapeutic drug and embolizing agent to the HCC which will cause tumor necrosis and tumor control, and preserve as much normal liver parenchyma as possible. But the shortcoming of TACE which could not be overcome by itself is unable to completely kill tumor cells, even if patients were treated with superselective TACE. The liver tumor has two blood
supplies unlike healthy liver tissue, the hepatic artery provides
almost all tumor blood supply and the portal vein provides
remains. The portal blood supply feeding mainly in the tumor
periphery, and the blood may flow into tumor via the portal vein in
the tumor periphery in a retrograde manner by following a
pressure gradient in tumor sinusoids after TACE. Accordingly,
tumor periphery could continue to grow with portal vein branch
feeding, thus result in disease progression after TACE.14,15

HCC patients are often found to have functional deficiency in
host adaptive immunity response and innate immunity response.16
Antitumor immunity mainly depends on cellular immune
response. Therefore, cellular immunity dysfunction is one of
the reasons why tumors are incurable, and easy to relapse or
metastasize. Many studies reported that CIK cells could suppress
the growth of HCC cells, boost the cellular immunity in HCC
patients.17,18 Many case analyses showed that CIK cell therapy
could enhance the efficacy of interventional treatment. For
advanced HCC patients or those who were unfit for surgery or
chemotherapy, CIK cell therapy could ameliorate symptoms,
reduce quality of life and prolong survival of patients.22,23
Many studies have illuminated that CIK cells possess strong
cytotoxicity, could kill drug-resistant HCC cells by inducing
apoptosis, and could produce IL-2, IL-6, IFN-γ and other
anti-tumor cytokines.22,23 The cellular immunity of HCC patients
is significantly impaired by anticancer drugs for TACE.14, TACE
combined with CIK cell infusion hereby become an important
treatment for HCC patients.

Because of high cost of CIK cells, it is difficult to conduct
randomized controlled clinical trials to evaluate the efficacy of CIK
cells in the adjuvant treatment of HCC after TACE. Thus, we
conducted this non-randomized concurrent control trial to
evaluate the efficacy of CIK cells on HCC after TACE. In the
present study, the median PFS and OS were significantly
increased for the patients received TACE combined with CIK cell
therapy. The median PFS increased by 6 months, the median OS
increased by 21 months. The times of TACE, ECOG
performance status, and CIK cell therapy were independent
prognostic factors of all patients. For patients adopted more than
one time of TACE before disease progression, there would be
low residual tumor burden which plays an important role in
prolonging PFS of HCC patients after TACE. For the patients with
ECOG performance status of 2–3, usually associated with
impaired immune function, CIK cell therapy did not contribute to
the survival benefit.

To sum up, adjuvant immunotherapy with CIK cells may
greatly improve efficacy of TACE on HCC, and plays an
important role in prolonging the PFS of HCC patients after TACE.

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