

Efficacy of RetroNectin-activated cytokine-induced killer cell therapy in the treatment of advanced hepatocellular carcinoma

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Abstract. The present study aimed to investigate the efficacy of RetroNectin-activated cytokine-induced killer cell (R-CIK) therapy in advanced hepatocellular carcinoma patients as compared with conventional chemotherapy, a comparison that has not yet been thoroughly studied. From January 2010 to October 2013, 74 patients with an initial diagnosis of advanced hepatocellular carcinoma were enrolled in the study. Patients were assigned to one of two treatment arms: patients in arm 1 (n=37) received R-CIK treatment as the first line therapy, whereas those in arm 2 (n=37) received chemotherapy as the first line treatment. The primary end point measured in this study was median overall survival (mOS). Median progression-free survival time (mPFS) and 1- and 2-year survival rates were recorded as secondary end points. Kaplan-Meier analysis was performed on all mOS and mPFS data, and treatment hazard ratios were established using the Cox proportional hazards model. The 1-year survival rate in treatment arm 1 was 42.47% vs. 24.89% in arm 2 (95% CI, 24.91-59.01% vs. 12.10-40.02%, P=0.066); the 2-year survival rates were 21.24 and 5.53% (95% CI, 4.60-45.86 vs. 0.46-21.06%, P=0.106) in arms 1 and 2, respectively; the mPFS in arm 1 was 4.37 vs. 3.90 ($\chi^2=0.182$, P=0.670) in arm 2; and the mOS in arm 1 was 14.03 months vs. 9.46 months ($\chi^2=4.406$, P=0.036) in arm 2. Calculations of univariate analyses of arm 1, R-CIK cycles ≥ 6 , KPS >70 , AFP ≤ 400 ng/ml, and findings of no vascular invasion and no extra-hepatic metastasis were potential predictive factors

(P<0.05). Calculations from multivariate analyses similarly identified these factors as potentially having predictive value (P<0.05). The main adverse effects of R-CIK therapy included fever and headache pain. R-CIK treatment may prolong mOS in advanced hepatocellular carcinoma patients compared with conventional chemotherapy. Patients who underwent ≥ 6 cycles of R-CIK, had KPS scores >70 , AFP ≤ 400 ng/ml, displayed no evidence of vascular invasion, and no extra-hepatic metastasis appeared to have longer survival times compared with other cohorts in the present study.

Introduction

Hepatocellular carcinoma (HCC), the most common of the hepatobiliary malignancies, ranks as the fifth most common type and the third primary cause associated with cancer mortality (1). Recent data indicates that the incidence of HCC appears to be increasing rapidly all over the world (2). Although the majority of cases are observed in developing nations, there is also evidence of a rising incidence of HCC in both North America and Europe (3,4). Despite aggressive research, the prognosis for HCC patients remains relatively poor, and a definitive cure remains elusive, particularly in advanced cases.

At present, partial hepatectomy remains a potential curative treatment for most early-stage HCC patients (solitary tumor ≤ 5 cm in size, or ≤ 3 tumors each ≤ 3 cm in size and no evidence of gross vascular invasion) (5-7). Although partial liver resection may achieve a 5-year survival rate of ~50-70%, HCC recurrence rates at 5 years have been reported to exceed 70% following such procedures (8-10). In addition, because of the limitations of preserved liver function, considerations of patient performance, and factors such as tumor size and position, $<30\%$ patients are deemed suitable candidates for partial resection surgery (11). Liver transplantation is another potentially curative therapeutic option for patients with early-stage HCC. However, the shortage of donor organs and the numerous factors that complicate finding, acquiring, and successfully transplanting a well-matched liver severely restrict the use of transplantation as a treatment alternative (5,12). In recent years, the techniques of local-regional ablation and embolization

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have provided additional local-regional treatment options for patients who are not suitable surgical candidates (13,14). However these therapies are not adequate for patients with advanced HCC.

Sorafenib, an oral multi-kinase inhibitor that suppresses tumor cell proliferation and angiogenesis, has been shown to effectively improve outcomes in cases of advanced or metastatic HCC. However, the impact of this drug is quite limited, as the mOS of advanced or metastatic HCC patients on sorafenib is only 6.5-10.7 months (15,16). Recently, the development of novel chemotherapy drugs such as Oxaliplatin, Capecitabine, and Gemcitabine among others has facilitated the development of systemic chemotherapy regimens for the treatment of HCC. However, this approach has so far only demonstrated modest improvements in outcomes, including an mOS of 6.47-12.2 months in the treatment of advanced HCC (17-19).

Because of the limitations of surgical and chemotherapeutic options, immunotherapy has gained steadily increasing attention as a treatment option for advanced HCC. In 1991, Schmidt-Wolf, IG *et al* identified a type of anti-tumor effector cells, known as cytokine-induced killer cells (CIK), that proliferate rapidly *in vitro*, possess strong antitumor activity against a broad spectrum of solid tumors, and cause minimal adverse effects when active in the host's body (20). As expected, the anti-tumor effect of CIK depends on the number of active CIK cells and on the overall functional quality of the cells, a factor that is particularly important when considering the techniques employed to culture CIK cells for therapeutic delivery. Recent studies have reported that RetroNectin can improve conglutination, extension, differentiation and proliferation of cultured cells, all of which contribute to more efficient stimulation of T cells (21-24). Our previous study also confirmed this (25). In the present study, the clinical effects of R-CIK therapy were compared with standard chemotherapy and the predictive factors governing the efficacy of R-CIK treatment were investigated in patients with advanced HCC.

Materials and methods

Patients. Between January 2010 and October 2013, 74 patients with an initial diagnosis of advanced hepatocellular carcinoma (AJCC criteria) were enrolled in this study. Patients were assigned to either arm 1 (n=37) to receive CIK treatment as a first line therapy, or arm 2 (n=37) to receive chemotherapy [Oxaliplatin (130 mg/m²) combined with Capecitabine (1,000 mg/m²)] as a first line treatment. The present study was approved by the Ethics Committee at The Affiliated Cancer Hospital of Zhengzhou University (Zhengzhou, China) and an approved consent form was signed by all the patients. For purposes of analysis, two subsets of HCC patients were identified. One subset consists of patients who had previously received local-regional treatments [e.g. transcatheter arterial chemoembolization (TACE), radiofrequency ablation (RFA), surgery] for early HCC, but who had subsequently developed distant metastasis and/or multiple intra-hepatic metastases (>3 nodules) that could not be effectively treated with local-regional treatments and where the patient could not reasonably be expected to pay the fee of sorafenib therapy. The other subset of patients identified had received no prior therapy and presented initially with distant metastasis and/or

multiple intra-hepatic metastases (>3 nodules) that could be effectively with local-regional treatments and for whom the fee of sorafenib therapy could not be paid. Both subsets of patients were included in the group considered to have advanced HCC and were enrolled in the study. The clinical characteristics of the 74 patients are detailed in Table I.

Study design and response. Patients were assigned to one of two treatment groups. Patients in arm 1 received R-CIK as a first line therapy, while patients in arm 2 received chemotherapies [Oxaliplatin (130 mg/m²) combined with Capecitabine (1,000 mg/m²)]. The primary endpoint of the study was mOS, and secondary endpoints were mPFS and 1- and 2-year survival rates. During treatment, patients received follow-up and reviews every 2 months to evaluate the changes in patients' conditions. The follow-up deadline was December 8, 2014. Responses to therapy were evaluated using RECIST 1.1 criteria (26). According to these criteria, clinical effects were divided into complete response (CR), partial response (PR), stable disease (SD) and progressive disease (PD). PFS was calculated from the first treatment to the time at which patients were found to meet the criteria for PD. OS was calculated from the time of their first treatment to the time of death or to the time the patient was re-censored at the last instance of contact with the live patient. Adverse reactions of these regimes were evaluated using the National Cancer Institute Common Toxicity Criteria 3.0 (NCI-CTC 3.0), accordingly, reactions were divided into grades 1-4 (27). In arm 1, when patients emerged as meeting the criteria for PD, R-CIK combined with chemotherapy (Oxaliplatin combined with Capecitabine) or R-CIK and/or best supportive care (BSC) was applied to these patients; when patients were identified to meet the criteria for CR/PR/SD, R-CIK or BSC would be applied to these patients. However, in arm 2, when patients met the criteria for PD, other chemotherapy methods and/or BSC would be applied to these patients; when patients in arm 2 were found to qualify as showing CR/PR/SD, chemotherapy or BSC was continued. Details of the courses of therapy in the two treatment arms are shown in Fig. 1.

Preparation of R-CIK. The method used for CIK cell preparation in the present study was slightly different from methods detailed in previous literature (25,28). Peripheral blood mononuclear cells (PBMCs) were collected from 50 ml peripheral blood draws taken from patients. These cells were coated with RetroNectin (10 µg/ml; Takara Biomedical Technology Co., Ltd, Beijing, China) and anti-CD3 antibody (5 µg/ml; catalog no. 7381803; GE Healthcare Bio-Sciences, Pittsburgh, PA, USA) for 24 h and then cultured in GT-T551 medium (Takara Bio Inc., Otsu, Japan), which contained recombinant human interleukin-2 (rhIL-2; 1,000 U/ml), interferon-γ (IFN-γ; 1,000 U/ml) and 5% inactive autogeneic plasma, at 37°C with 5% CO₂ for 4 days. Then the cells were cultured with fresh IL-2 and 2% inactive autogeneic plasma-containing medium for 5 days. At day 15, R-CIKs were harvested and analyzed for phenotype. All the products were free of bacterial, mycoplasma, and fungal contamination. The endotoxin level was <5 EU in all samples.

Phenotypic assessment of R-CIK. R-CIKs (1x10⁶) and PBMCs (1x10⁶) were harvested and were double stained with 10 µl

Table I. Patient characteristics.

Characteristic	Arm 1	Arm 2	P
Number of patients	37	37	
Age (years)			0.227
<60	26	21	
≥60	11	16	
Gender			0.207
Male	29	33	
Female	8	4	
Diameter of tumor (cm)			0.321
≥5, <7	10	14	
>7	27	23	
KPS (scores)			0.227
>70	21	26	
≤70	16	11	
Child-Pugh			0.075
A+B	27	33	
C	10	4	
AFP (ng/ml)			0.480
>400	20	23	
≤400	17	14	
Hepatitis B			0.553
Yes	31	29	
No	6	8	
Vascular invasion			0.566
Yes	15	13	
No	21	24	
Cirrhosis			0.183
Yes	30	25	
No	7	12	
Extrahepatic metastasis			0.480
Yes	23	20	
No	14	17	
Ascites			0.407
Yes	10	7	
No	27	30	
Once TACE			0.344
Yes	20	24	
No	17	13	
Once RFA			0.295
Yes	8	12	
No	29	25	
Once surgery			0.626
Yes	12	14	
No	25	23	
Albumin (g/l)			0.782
<35	8	9	
≥35	29	28	
Bilirubin (μmol/l)			0.483
>ULN	18	15	
≤ULN	19	22	

Table I. Continued.

Characteristic	Arm 1	Arm 2	P
ALT (U/l)			0.451
>ULN	13	10	
≤ULN	24	27	
AST (U/l)			0.639
>ULN	17	15	
≤ULN	20	22	
γ-GGT (U/l)			0.632
>ULN	24	22	
≤ULN	13	15	
PT (s)			0.812
>ULN	15	14	
≤ULN	22	23	
PLT (/l)			0.259
>LLN	27	31	
≤LLN	10	6	
Median R-CIK cycles	5 (3-24)	0	
KPS, Karnofsky; AFP, alpha-fetal protein; TACE, transarterial chemoembolization; RFA, radiofrequency ablation; ULN, upper limit of normal; ALT, alanine transaminase; AST, aspartate transaminase; γ-GGT, gamma glutamyl transpeptidase; PT, prothrombin time; PLT, platelet; LLN, lower limit of normal.			
fluorescein isothiocyanate (FITC) CD3 (catalog no., 555342) and phycoerythrin (PE) CD4 (catalog no., 555347), CD8 (catalog no., 555635), CD16 (catalog no., 555408) and CD56 (catalog no., 555517) (BD Biosciences, San Jose, CA, USA), all at a 1:200 dilution. Samples were incubated at 4°C for 30 min, then washed twice with PBS and re-suspended in 500 ml PBS. Fluorescence was detected by FACS Calibur flow cytometer (BD Biosciences) and data on 10,000 cells were acquired and analyzed. Propidium iodide and annexin V-FITC (BD Biosciences) were used to measure viability and apoptosis, according to the manufacturer's protocol.			
<i>Cytotoxicity assessment of R-CIK.</i> At the end of cell expansion, R-CIKs' anti-tumor activity was tested using an overnight cytotoxicity assay with K-562 (chronic myeloid leukaemia) cell line cells used as targets. The cytotoxic activity of cells was investigated in a lactate dehydrogenase (LDH) release assay. This non-radioactive assay is a colorimetric alternative to the ⁵¹ Cr release assay and quantitatively measures LDH that is released upon cell lysis in the same way that ⁵¹ Cr is released. Every experiment, at each effector cell concentration, was performed in a triplicate set of wells and, the mean value was calculated.			
<i>Statistical analysis.</i> SPSS software, version 17.0(SPSS, Inc., Chicago, IL, USA) was used to perform the statistical analysis. Data for phenotypic analysis of R-CIK cells and cytotoxicity were analyzed by independent sample <i>t</i> -tests. For survival time analysis, the Kaplan-Meier method was used. Prognostic factors of survival time were analyzed using log-rank test and			

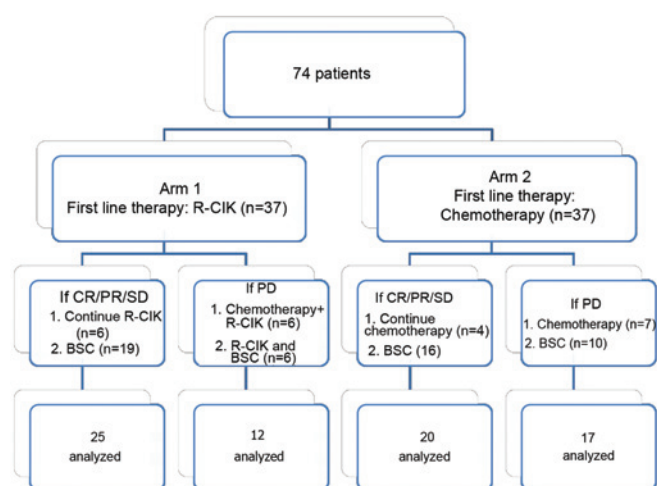


Figure 1. Flow chart describing patient allocation into treatment groups. Patients were assigned to two treatment groups: arm 1 and arm 2. R-CIK, RetroNectin-activated cytokine-induced killer cell; CR, complete remission; PR, partial remission; SD, stable disease; BSD, best standard care; PD, progressive disease.

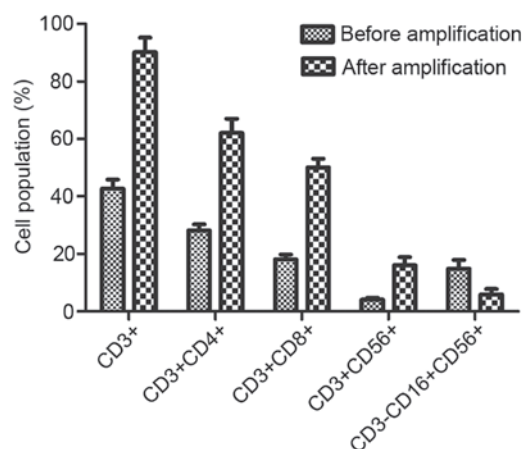


Figure 2. Phenotype of peripheral blood mononuclear cells and RetroNectin-activated cytokine-induced killer cell therapy.

multivariable analysis. For all data, $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Phenotypic analysis of R-CIKs. Phenotypic analysis of R-CIKs in the patients prior to culture and after 15 days of culture indicated that the percentages of CD3+, CD3+/CD4+, CD3+/CD8+ and CD3+/CD56+, increased from 42.67 ± 3.21 , 28.12 ± 2.16 , 18.13 ± 1.66 and $4.12 \pm 0.56\%$ to 90.21 ± 5.02 , 62.01 ± 5.01 , 50.13 ± 3.03 and $24.03 \pm 2.81\%$ respectively, (all $P < 0.05$). In contrast, the population of CD3-/CD16+/CD56+ cells reduced from 14.87 ± 2.99 to $5.89 \pm 1.87\%$ ($P < 0.05$). The details are presented in Fig. 2.

Cytotoxicity of R-CIK in vitro. A sample of each R-CIK expansion obtained from a total of 37 patients was tested for cytotoxicity against K-562, an NK-sensitive leukemia target cell line. At effector to target (E/T) cell ratios of 40:1, 20:1

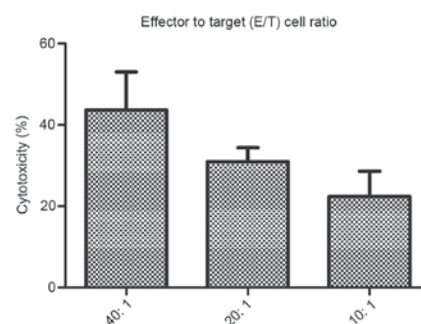


Figure 3. Mean RetroNectin-activated cytokine-induced killer cell cytotoxicity for K-562 cell line.

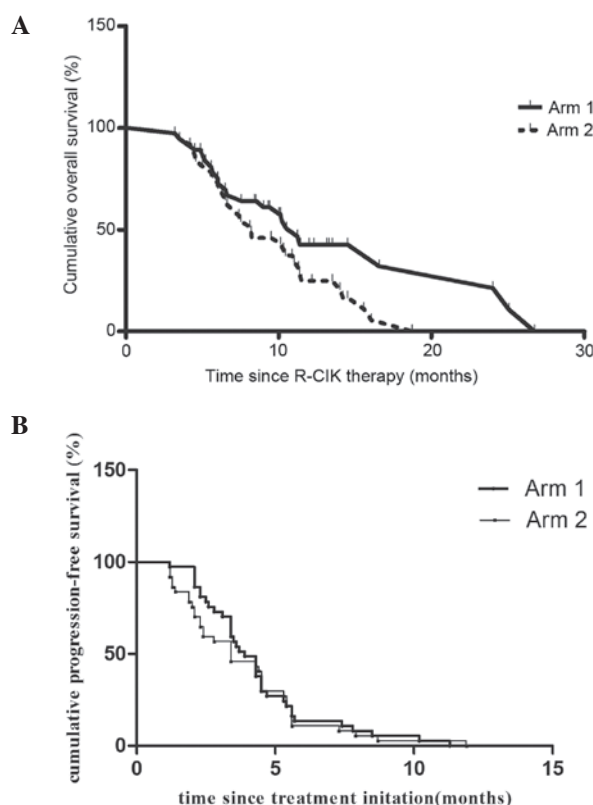


Figure 4. (A) Overall survival of patients in arm 1 and arm 2. (B) Progression free survival of patients in arm 1 and arm 2. R-CIK, RetroNectin-activated cytokine-induced killer cell.

and 10:1 the median levels of cytotoxicity were $43.66 \pm 9.36\%$, $30.95 \pm 3.49\%$ and $22.39 \pm 6.24\%$, respectively. The details are presented in Fig. 3.

Treatment outcomes. Follow-up with all patients occurred from January 2010 to October 2013, the outcomes observed in arm 1 patients were significantly improved compared with the outcomes in arm 2 patients. Specially, the mOS of arm 1 vs. arm 2 was 14.03 vs. 9.46 months ($\chi^2 = 4.406$ $P = 0.036$) (Fig. 4A). The 1-year survival rate was 42.47 vs. 24.89% (95% CI 24.91-59.01% vs. 12.10-40.02%, $P = 0.066$), the 2-year survival rate was 21.24 vs. 5.53% (95% CI 4.60-45.86% vs. 0.46-21.06%, $P = 0.106$), and the mPFS of arm 1 vs. arm 2 was 4.37 vs. 3.90 ($\chi^2 = 0.182$ $P = 0.670$) (Fig. 4B), indicating no significant differences in these measures. Although the mPFS,

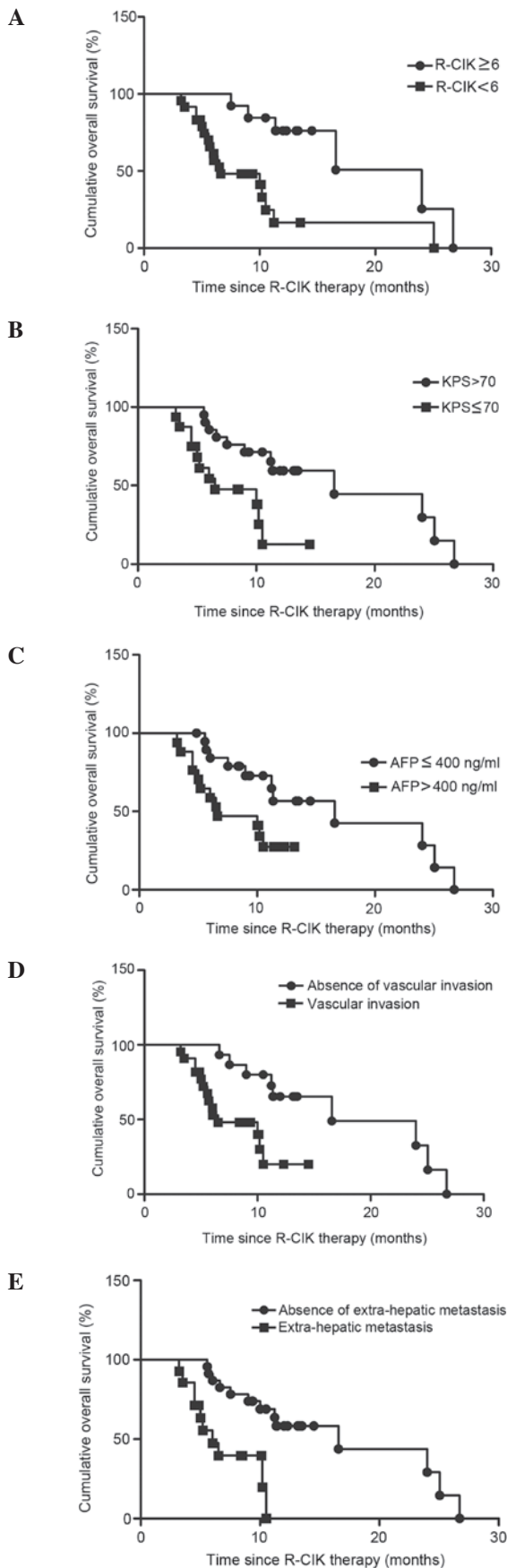


Figure 5. (A) OS of patients in arm 1 based on R-CIK cycles; (B) OS of patients in arm 1 based on KPS scores; (C) OS of patients in arm 1 based on AFP; (D) OS of patients in arm 1 based on vascular invasion or not; (E) OS of patients in arm 1 based on extra-hepatic metastasis or not. R-CIK, RetroNectin-activated cytokine-induced killer cell; OS, overall survival; AFP, alpha-feto protein; KPS, Karnofsky.

Table II. Univariate analysis.

Variable	Median OS (months)	P	Median PFS (months)	P
Age (years)		0.697		0.468
<60	13.674		4.546	
≥60	10.269		3.955	
Gender		0.605		0.517
Male	14.440		4.507	
Female	9.390		3.875	
Diameter of tumor (cm)		0.406		0.508
≥5, <7	14.086		4.760	
>7	11.244		4.226	
KPS (scores)		0.004		0.401
>70	17.014		4.638	
≤70	7.964		4.019	
Child-Pugh		0.145		0.339
A+B	15.057		4.578	
C	8.151		3.810	
AFP (ng/ml)		0.026		0.125
>400	8.270		3.763	
≤400	16.768		4.833	
Hepatitis B		0.991		0.961
Yes	13.327		4.217	
No	13.868		4.400	
Vascular invasion		0.004		0.304
Yes	8.423		4.055	
No	18.300		4.833	
Cirrhosis		0.283		0.669
Yes	8.762		3.943	
No	14.781		4.470	
Extrahepatic metastasis		0.001		0.116
Yes	7.030		3.657	
No	16.928		4.804	
Ascites		0.979		0.508
Yes	9.878		4.226	
No	14.064		4.760	
Once TACE		0.731		0.263
Yes	14.452		4.745	
No	10.142		3.929	
Once RFA		0.720		0.331
Yes	13.543		5.100	
No	11.943		4.169	
Once surgery		0.258		0.227
Yes	16.149		5.033	
No	11.215		4.052	
Albumin (g/l)		0.605		0.228
<35	9.391		4.131	
≥35	14.444		5.238	

Table II. Continued.

Variable	Median OS (months)	P	Median PFS (months)	P
Bilirubin ($\mu\text{mol/l}$)		0.792		0.396
>ULN	10.553		4.100	
\leq ULN	13.571		4.656	
ALT (U/L)		0.283		0.355
>ULN	11.271		4.113	
\leq ULN	16.081		4.846	
AST (U/L)		0.625		0.310
>ULN	10.681		4.070	
\leq ULN	13.161		4.724	
γ -GGT (U/L)		0.787		0.238
>ULN	13.743		3.846	
\leq ULN	10.104		4.654	
PT (s)		0.620		0.404
>ULN	10.636		4.136	
\leq ULN	13.027		4.713	
PLT (/L)		0.238		0.657
>LLN	17.092		4.481	
\leq LLN	12.913		4.070	
R-CIK cycles		0.002		0.154
≥ 6	19.317		5.077	
< 6	10.173		3.988	

KPS, Karnofsky; AFP, alpha-fetal protein; TACE, transarterial chemoembolization; RFA, radiofrequency ablation; ULN, upper limit of normal; ALT, alanine transaminase; AST, aspartate transaminase; γ -GGT, gamma glutamyl transpeptidase; PT, prothrombin time; PLT, platelet; LLN, lower limit of normal.

1-year survival rates, and 2-year survival rates demonstrated no significant differences between the two treatment arms, the mOS of arm 1 was significantly prolonged when compared with arm 2. Therefore, patients with advanced HCC who could not receive local-regional treatments benefited significantly from R-CIK treatment.

Prognosis factors of R-CIK treatment based on mOS. Univariate analyses of arm 1 revealed that receiving ≥ 6 cycles of R-CIK (Fig. 5A), a KPS > 70 (Fig. 5B), AFP ≤ 400 ng/ml (Fig. 5C), absence of vascular invasion (Fig. 5D), and absence of extra-hepatic metastasis (Fig. 5E) were all potential predictive factors ($P < 0.05$), (Table II). Multivariate analyses similarly identified these factors as potentially predictive ($P < 0.05$) (Table III).

Adverse events. The incidences of adverse treatment effects are described in Table IV. None of the patients in arm 1 failed to complete immunotherapy or were ruled out because of side effects. No severe adverse effects (grade 3 or 4) were associated with R-CIK therapy. Common side effects of immunotherapy (grade 1 or 2) included fever and headache pain. None of the patients in arm 2 failed to complete chemotherapy or were

ruled out because of side effects. Common side effects of chemotherapy (grade 1 or 2) included nausea, leukopenia, thrombocytopenia and liver dysfunction. There were 4 patients that developed grade 3 or 4 side effects: Two patients developed fevers and two were found to have leukopenia.

Discussion

At present, advanced HCCs are not sufficiently treated with traditional chemotherapy or radiotherapy. And while local-regional treatments may be appropriate for cases of early HCC, they are not suitable for patients with advanced HCC. Fortunately, recent developments in immunology have given researchers and clinical physicians the option of utilizing immunotherapy as a significant component of cancer treatment (29).

CIK cell therapy is emerging as an important form of adoptive immunotherapy thanks to its high amplification efficiency and strong anti-tumor activity. CIK cell therapy represents a realistic novel option in the field of cancer therapy as it consistently demonstrates strong anti-tumor activity and improves the overall survival time of cancer patients when used alone, or when combined with other conventional therapies. Since 1991, CIK cell therapy has been evaluated as an adoptive immunotherapy for cancer patients in a number of clinical trials, including in patients with HCC (28,30-33). For example, Pan *et al* (34) reported that CIK cell treatment as an adjuvant therapy for postoperative hepatocellular carcinoma patients could increase overall survival rates compared with surgery alone. Similarly, Yu *et al* (31) reported that adding CIK treatment could prolong the overall survival time and progression-free survival time of HCC patients found to be unsuitable for surgical treatment in 2014. In 2015, Lee *et al* reported (35) that adjuvant immunotherapy with CIK cells increased recurrence-free and overall survival combined with surgical resection, radiofrequency ablation, and percutaneous ethanol injection in a multi-center, randomized, open-label, phase 3 trial. These reports indicate that CIK cell immunotherapy has significant potential benefits for cancer patients. However, the specific question of whether CIK cell therapy can improve survival time of patients with advanced HCC remained unclear.

The effectiveness of CIK cell therapy depends on the quality and quantity of CIK cells obtained for therapeutic administration. In the CIK cell preparations in the present study, the method of culture was slightly different from methods employed in previous literature and it was possible to obtain more of the CD3+/CD56+ cellular subset on day 15 than common CIK cells preparation methods achieve (28). RetroNectin (RN) was added to the culture media in our CIK cell preparation (called R-CIK), which improves the conglutination, extension, and differentiation of these cells (25). Our results indicate that the percentages of CD3+, CD3+/CD4+, CD3+/CD8+ and CD3+/CD56+ cells were significantly increased after stimulation and expansion in culture (25). In addition, there was a significant decrease in the CD3-/CD16+/CD56+ population. It is widely accepted that the increase in CD4+ and CD8+ T cells during CIK expansion is crucial to tumor immunity. CD3+/CD56+ cells, which comprise a rare population of cells in normal peripheral

Table III. Multivariate analysis.

Parameters	Hazard ratio	95% confidence interval	P-value
R-CIK (≥ 6 cycles vs. 6 cycles)	0.224	0.079-0.632	0.005
AFP (ng/ml) (>400 vs. ≤ 400)	0.363	0.142-0.924	0.034
KPS (>70 scores vs. ≤ 70 scores)	0.265	0.102-0.687	0.006
Vascular invasion (yes vs. no)	0.227	0.078-0.661	0.007
Extrahepatic metastasis (yes vs. no)	0.201	0.074-0.546	0.002

R-CIK, RetroNectin-activated cytokine-induced killer cell; OS, overall survival; AFP, alpha-feto protein; KPS, Karnofsky.

Table IV. Adverse events distribution.

Side effects	Arm 1		Arm 2	
	Grade 1/2	Grade 3/4	Grade 1/2	Grade 3/4
Fever	15	0	8	2
Headache pain	8	0	7	0
Nausea	5	0	15	0
Leukopenia	30	0	33	2
Liver dysfunction	20	0	25	0
Diarrhea	2	0	4	0
Thrombocytopenia	10	0	7	0
Anemia	5	0	7	0

blood, are also significantly increased after stimulation and expansion. These may be the most important cells for the anti-cancer effect of CIK treatment (36) as they have shown strong anti-cancer activity in a variety of malignant tumor cell lines *in vitro* (20,25,37). Therefore, we have good reason to hypothesize that our improved methods have allowed us to obtain R-CIK cells with particularly strong anti-tumor activity for transferring them to patients.

Although the present study failed to show a significant difference in mPFS between the two treatment arms, R-CIK treatment did significantly prolonged the mOS of advanced HCC patients compared with the chemotherapy arm. In the calculations from univariate analyses of arm 1, R-CIK cycles ≥ 6 , KPS >70 , AFP ≤ 400 ng/ml, the absence of vascular invasion and a lack of extra-hepatic metastasis were all potential predictive factors ($P < 0.05$). In the calculations from multivariate analyses, these factors were also found to have potential predictive value ($P < 0.05$). These results indicate that the likely effects of R-CIK treatment can be reasonably well predicted based on preliminary laboratory tests and imaging reports.

In conclusion, the present study indicated R-CIK cell therapy can improve prognosis in advanced HCC and increasing the number of cycles of R-CIK cell therapy is likely to result in additional benefits. Discrepancies versus previous studies of CIK therapy may be attributed to the differences in stimulation and expansion methods, genetic and environmental backgrounds, staging systems, and sample size. It will certainly be necessary to carry out numerous additional

studies in order to optimize techniques and determine the full range of application for this type of therapy. At the same time, the combination of R-CIK with conventional chemotherapy, as well as R-CIK combined with Sorafenib also clearly merit exploration.

R-CIK treatment can prolong mOS of advanced hepatocellular carcinoma patients compared with conventional chemotherapy alone. Patients who undergo >6 cycles of R-CIK, have a KPS >70 , an AFP ≤ 400 ng/ml, no vascular invasion and no extra-hepatic metastases may have longer survival times in comparison with other patients.

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