

**HARW Beta 15.0**  
**Dummy Title seventeen**  
--Manuscript Draft--

<b>Manuscript Number:</b>	
<b>Full Title:</b>	Dummy Title seventeen
<b>Article Type:</b>	Original Article
<b>Corresponding Author:</b>	* Amy Author Springer Heidelberg, GERMANY
<b>Corresponding Author Secondary Information:</b>	
<b>Corresponding Author's Institution:</b>	Springer
<b>Corresponding Author's Secondary Institution:</b>	
<b>First Author:</b>	* Amy Author
<b>First Author Secondary Information:</b>	
<b>Order of Authors:</b>	* Amy Author
<b>Order of Authors Secondary Information:</b>	
<b>Author Comments:</b>	Test
<b>Suggested Reviewers:</b>	

# Effective Activity of Cytokine Induced Killer Cells against Hepatocellular carcinoma including Tumor-initiating cells

Xiaoxiang Rong<sup>1\*</sup>, Fang Wei<sup>2\*</sup>, Aimin Li<sup>1</sup>, Dong Xiao<sup>2§</sup>, Rongcheng Luo<sup>1§</sup>

<sup>1</sup>Department of Oncology, Nanfang Hospital, Southern Medical University,  
Guangzhou 510515, Guangdong Province, China;

<sup>2</sup>Cancer Research Institute, Southern Medical University, Guangzhou 510515,  
Guangdong Province, China.

## Abstract

Tumor-initiating cells(TICs) are a subpopulation of chemoresistant tumor cells that have been shown to cause tumor recurrence. Targeting and eliminating of TICs are therefore priorities for the development of new therapeutic paradigms. Much promise lies in adoptive immunotherapy. Cytokine-induced killer (CIK) cells are heterogeneous ex vivo-expanded T lymphocytes, with a mixed T-NK phenotype. It represents a realistic new option in the field of Hepatocellular carcinoma(HCC) immunotherapy. In the very recent years, Large clinical trials demonstrated that CIK cells could improve the Progression Free Survival (PFS) and Overall Survival(OS) in patients with HCC. By the same time, several studies reported that CIK cells were

capable of clearing cells with stemness features in Lymphoma, Melanoma, Bone and Soft-Tissue Sarcomas. Based on the findings above mentioned, we hypothesized that CIK cells could eliminate the tumor-initiating cells, improving the PFS and OS of patients with HCC when combined with radiofrequency ablation(RFA) or transcatheter arterial chemoembolization(TACE).

**Keywords:** Hepatocellular carcinoma, Cytokine-induced killer cells, Tumor-initiating cells.

## Introduction

Hepatocellular carcinoma(HCC) is one of the deadliest cancers, mainly due to its high rate of recurrence, which can be as high as 70% following conventional treatments such as surgical resection, arterial embolization , and radiofrequency ablation[1,2]. HCC, like other cancer types, is composed of phenotypically and functionally diverse cell types. It seems that as hypothesized for other tumors, HCC includes a small cell subpopulation referred to as cancer stem cells or tumor-initiating cells(TICs) endowed with the stemness features that sustain drug resistance and disease relapse[3,4]. This challenging clinical scenario demands new therapeutic approaches, ideally the ones that are able to target tumor-initiating cells(TICs).

Much promise lies in adoptive immunotherapy for the treatment of metastatic HCC. Cytokine-induced killer(CIK) cells are heterogeneous ex vivo-expanded T

lymphocytes, with a mixed T-NK phenotype, able to exert a wide MHC-unrestricted antitumor activity against both solid and hematologic malignancies[5]. CIK cells can be massively expanded from peripheral blood mononuclear cells(PBMC) cultured with the timed addition of IFN- $\gamma$ , Ab-anti-CD3, and interleukin(IL)-2 through simple standardized culture conditions[6]. Initial clinical trial resulting in various tumor setting are encouraging, including HCC, more and more evidence indicating that the combination of CIK with RFA for HCC patients could improve PFS, and be helpful in the prevention of the recurrence[7,8]. Whereas, whether or not the tumor-killing ability of CIK cells affects the subpopulation of HCC TICs is completely un explored.

Currently, clear identification of HCC TICs is intensely debated. Several membrane molecules or genes have been proposed as putative markers, such as CD133[3], EpCAM[9], CD13[10], CD44[11], CD90[12], CD24[4], but agreement remains elusive. While the identification and isolation of a pure population of slow-dividing TICs will serve as a valuable tool to confirm whether the CIK cells were capable of targeting and clearing the TICs. As we know, quiescence prevents stem cell exhaustion in normal tissues[21]. Only quiescent stem cells retain the ability to resume proliferation when compared with other nonproliferative status (terminal differentiation/senescence)[22]. Thereby, quiescent stem cells and its reversibility become defining parameters of stem cells. PKH-26, a fluorescent dye, could be equally distributed between daughter cells upon division, leading to the halving of the fluorescence intensity with every cell division. The intensity of PKH-26 staining correlates with the cell division and then discriminates the highly cycling from the

slowly dividing cells(putative TICs). Although PKH-26 dye has not been used to enrich TICs in HCC, more and more evidence demonstrated the feasibility to take advantage of fluorescent dye as PKH-26 to enrich for slow-dividing cells retaining high capability to self-renew to represent TICs[14,15,23,24]. In Kusumbe 's study, they demonstrated that stem cell activity is enriched in the quiescent fraction of a tumor that shows the capability to revert to a state of self-renewal and regeneration though PKH-26 label retention[24]. Similar findings were found in glioblastoma and nasopharyngeal carcinoma[14,15].

Here, we hypothesize that CIK cells can clear the HCC TICs and induce an acquired immune response that prevents tumor relapse. To highlight the HCC TICs, we could use the PKH-26 fluorescent dye to identify slow-cycling PKH26+ cells, and sorted them on the basis of PKH-26 staining. This approach may, therefore, be applied to greatly enhance the effectiveness of conventional cancer therapies in a variety of settings and represents a novel means to directly target tumor-initiating cells.

## **Hepatocellular carcinoma cells and Tumor-initiating cells**

The hepatocellular carcinoma cell population consists of mature hepatocellular carcinoma cells and TICs. TICs are important for drug resistance and disease relapse. One generally accepted theory is that HCC is maintained by TICs, which are quiescent and do not respond to cell cycle-specific cytotoxic agents used to treat HCC. Thus, it is important to isolate the TICs for further investigate. Cheng Qian and colleagues constructed a lentiviral vector containing human Nanog promoter to drive the

expression of the green fluorescent protein(GFP) reporter gene. Applying this system ,they successfully isolated a small subpopulation of Nanog-positive cells and demonstrated that these cells represent genuine TICs[13]. therefore, a new approach to HCC therapy might focus on specific targeting of these populations.

## **CIK cells and HCC treatment**

The clinical activity of adoptive immunotherapy with CIK cells against solid tumors has been described in several clinical trials during the last decade, with a marked increment in their frequency in the very recent years. In the field of HCC, a very interesting perspective is offered by the possibility of using CIK cells as adjuvant therapy. There are several large studies reported a beneficial effect of adjuvant CIK cells. In the first one, Went et al. randomized 85 patients to receiving the infusion of CIK cells through the hepatic artery, or no adjuvant therapy, a significant increase in circulating CIK cells was reported, along with a reduction in disease recurrence rates at 1year(8.9%) and 18 months(15.6%) compared with controls(30 and 40%)[16]. In the second study, Huang et al. randomized 174 patients to two groups, 85 patients received CIK transfusion after minimally invasive therapy, while 89 patients in the control group were treated with minimally invasive therapy alone. Compared with the control group, patients in the CIK group had significantly longer OS (56 vs. 31mo) and PFS(17 vs. 10mo)[8]. In the third study, Pan ke, et al. infused more than 4 courses of autologous CIK cells in 204 patients after surgery, leaving a group of 206 patients without adjuvant therapy. The reported OS was significantly higher for patients

1 treated with CIK cells compared with controls[17]. Another important results were  
2  
3 reported by Cui J et al. in a very recent prospective clinical trial with 62 patients  
4  
5 affected by HCC. Patients were randomized to treatment with RFA or a combination  
6  
7 of CIK cells. The trial reported significantly improved PFS in patients treated with CIK  
8  
9 cells compared with controls[7]. Overall, these trials certainly demonstrated the  
10  
11 feasibility and the high safety level of immunotherapy strategies with CIK cells, along  
12  
13 with the existence of promising antitumor activity against HCC.  
14  
15  
16  
17  
18  
19  
20  
21

## 22 **CIK cells and TICs**

23  
24  
25 Therapies designed specifically to target the TICs may provide a dramatic  
26  
27 improvement in life expectancy for patients with cancers who are prone to relapse.  
28  
29 Recently, several studies reported that CIK cells were capable of clearing  
30  
31 tumor-initiating cells(TICs). In the first study, Christopher H, et al. generated CIK cells  
32  
33 infected with oncolytic viruses as a therapeutic strategy against minimal disease  
34  
35 states of lymphomas characterized by the persistence of cancer cells that display  
36  
37 stem cell-like properties and resistance to conventional therapies. They found that  
38  
39 the CIK cells were capable of trafficking to and targeting residual cancer cells[18]. In  
40  
41 the second one, Loretta Gammaitoni, et al. constructed a lentiviral vector encoding  
42  
43 the enhanced green fluorescence protein(eGFP) under expression control of the Oct4  
44  
45 promoter. And putative TICs was assessed by Oct4 promoter activity. They found that  
46  
47 CIK cells against TICs was comparable to differentiated cancer cells in Melanoma[19].  
48  
49  
50  
51  
52  
53  
54  
55  
56  
57  
58  
59 Similar research demonstrated the powerful tumor-killing capacity of CIK cells and  
60  
61  
62  
63  
64  
65

their ability to clear the TICs in Bone and Soft-Tissue Sarcomas[20]. In conclusion, CIK cells represent a realistic new option for adoptive immunotherapy targeting TICs. While the tumor-killing ability of CIK cells affects the subpopulation of HCC TICs is completely unexplored. A crucial issue would be the possibility to investigate the efficacy of CIK cells against HCC TICs.

## Hypothesis

Based on these concepts, we inferred that CIK cells could eliminate the tumor-initiating cells, improve the PFS and OS of patients with HCC when combined with RFA or TACE.

## Future experimental practice

Whether the hypothesis is valid, it should firstly be testified in HCC cell lines in vitro. These cells will be cultured with or without CIK cells in different proportion for 24 hours and then the ratio of PKH26+ cells will be examined, and the stemness genes such as CD44, CD90, CD133, EpCAM, Nanog should be detected by cytometry. Sphere forming assay will also be necessary. In vivo, NOD/SCID mice will be injected orthotopically with  $10^6$  HCC cells, and then these mice will be treated with CIK cells in different proportions based on groups. After euthanizing the animals, the tumor will be weighted. The HE staining and the stemness genes detection will be performed after the tumor recovered and fixed in order to detect the proportion of the TICs(Fig.1).



1 In order to examine whether the CIK cells could enhanced the antitumor effect of  
2  
3 radiotherapy or chemotherapy. The experiment should be performed in two parts.  
4  
5 For the in vitro assay, CCK8 or MTT assay will be employed to evaluate the cytotoxic  
6  
7 activity of oxaliplatin/radiation, CIK cells, and the oxaliplatin/radiation plus CIK cells  
8  
9 against HCC cells in vitro. HCC cells will be treated for 24 hours based on groups, and  
10  
11 then the HCC cells will be collected, clone formation assay and cell cycle assay will be  
12  
13 performed. For the in vivo assay. HCC cells will be injected into NOD/SCID mice  
14  
15 orthotopically to establish a tumor-bearing mice model. On the day 14, nomal saline,  
16  
17 oxaliplatin/radiation, CIK cells, and the combination therapy will be administered  
18  
19 respectively. After euthanizing the animals, the tumor will be weighted. The HE  
20  
21 staining will be performed and the Ki-67, BrdU will be detected using  
22  
23 immunohistochemical staining.  
24  
25  
26  
27  
28  
29  
30  
31  
32  
33  
34  
35  
36  
37  
38  
39  
40  
41  
42  
43  
44  
45  
46  
47  
48  
49  
50  
51  
52  
53  
54  
55  
56  
57  
58  
59  
60  
61  
62  
63  
64  
65



Fig1.Technology Roadmap

## Future clinical practice

Whether the hypothesis is valid , well-designed, double blinded, randomized and controlled clinical trials should be done to verify its role on the treatment of HCC. Patients with HCC **will receive** at least four courses of autologous CIK transfusion after minimally invasive therapy(Fig.2). For the preparation of CIK cells, briefly, 50 ml of heparinized peripheral blood **will be** obtained from the HCC patients. PBMCs **will be** separated and resuspended in fresh serum-free X-VIVO 15 medium containing 1000U/ml IFN- $\gamma$  and then incubated at 37°C in a humidified atmosphere containing 5% CO<sub>2</sub> for 24H. Then, 100ng/ml mouse anti-human CD3 monoclonal antibody, 100U/ml recombinant human IL-2 **will be** added every 2 days. the CIK cells **will be** harvested at

14 days. Before they were transfused, they **will be** assessed for viability by the dye exclusion test and checked for the possible contamination by bacteria, fungi and endotoxins. Kaplan-Meier and Cox regression analyses **will be** used to explore differences in PFS and OS between two groups. And this method can improve the PFS and OS in patients with HCC.

In order to identify whether CIK cells have the ability to eliminate the HCC TICs in clinical settings, human HCC tissues should be obtained from surgical specimens. Primary HCC cell cultures should be performed, and TICs will be marked with PKH-26 or GFP-Nanog. These cells cultured with or without autologous CIK cells in different proportion for 24 hours and then the ratio of PKH26+ cells or GFP+ cells **will be** examined. For the in vivo experiments, NOD/SCID mice **will be** injected orthotopically with HCC cells marked with GFP-Nanog, and then these mice **will be** treated with autologous CIK cells in different proportions based on groups. After euthanizing the animals, the tumor **will be** weighted. The HE staining and the stemness genes, GFP detection **will be** performed in order to detect the proportion of the TICs.

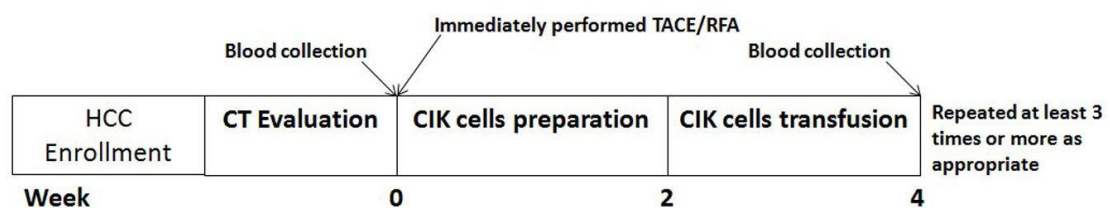


Fig2. Diagram of study procedures for combined therapy with CIK cells and minimally invasive therapies

## Conclusion

1 In conclusion, our hypothesis suggests a strategy to target TICs with CIK cells as  
2  
3 favorable candidates for clinical trials in HCC patients. The use of CIK cells either  
4  
5 independently or in synergism with other therapeutic strategies might be more  
6  
7 effective in destroying both tumor bulk and TICs.  
8  
9

## 10 11 12 13 14 **Conflict of interest** 15

16 The authors have no conflicts of interest to declare.  
17  
18  
19  
20  
21  
22  
23  
24  
25  
26  
27  
28  
29  
30  
31  
32  
33  
34  
35  
36  
37  
38  
39  
40  
41  
42  
43  
44  
45  
46  
47  
48  
49  
50  
51  
52  
53  
54  
55  
56  
57

## 58 **REFERENCES** 59 60 61 62 63 64 65

- [1]. Llovet, J.M. and J. Bruix, Molecular targeted therapies in hepatocellular carcinoma. *Hepatology*, 2008. 48(4): p. 1312-27.
- [2]. Xu, X.L., et al. , The properties of tumor-initiating cells from a hepatocellular carcinoma patient's primary and recurrent tumor. *Carcinogenesis*, 2010. 31(2): p. 167-74.
- [3]. Ma, S., et al. , Identification and characterization of tumorigenic liver cancer stem/progenitor cells. *Gastroenterology*, 2007. 132(7): p. 2542-56.
- [4]. Lee, T.K., et al. , CD24(+) liver tumor-initiating cells drive self-renewal and tumor initiation through STAT3-mediated NANOG regulation. *Cell Stem Cell*, 2011. 9(1): p. 50-63.
- [5]. Mesiano, G., et al. , Cytokine-induced killer (CIK) cells as feasible and effective adoptive immunotherapy for the treatment of solid tumors. *Expert Opin Biol Ther*, 2012. 12(6): p. 673-84.
- [6]. Lu, P.H. and R.S. Negrin, A novel population of expanded human CD3+CD56+ cells derived from T cells with potent in vivo antitumor activity in mice with severe combined immunodeficiency. *J Immunol*, 1994. 153(4): p. 1687-96.
- [7]. Cui, J., et al. , Combination of radiofrequency ablation and sequential cellular immunotherapy improves progression-free survival for patients with hepatocellular carcinoma. *Int J Cancer*, 2014. 134(2): p. 342-51.
- [8]. Huang, Z.M., et al. , Cytokine-induced killer cells in combination with transcatheter arterial chemoembolization and radiofrequency ablation for hepatocellular carcinoma patients. *J Immunother*, 2013. 36(5): p. 287-93.

- [9]. Yamashita, T., et al. , EpCAM-positive hepatocellular carcinoma cells are tumor-initiating cells with stem/progenitor cell features. *Gastroenterology*, 2009. 136(3): p. 1012-24.
- [10]. Haraguchi, N., et al. , CD13 is a therapeutic target in human liver cancer stem cells. *J Clin Invest*, 2010. 120(9): p. 3326-39.
- [11]. Zhu, Z., et al. , Cancer stem/progenitor cells are highly enriched in CD133+CD44+ population in hepatocellular carcinoma. *Int J Cancer*, 2010. 126(9): p. 2067-78.
- [12]. Yang, Z.F., et al. , Significance of CD90+ cancer stem cells in human liver cancer. *Cancer Cell*, 2008. 13(2): p. 153-66.
- [13]. Shan, J., et al. , Nanog regulates self-renewal of cancer stem cells through the insulin-like growth factor pathway in human hepatocellular carcinoma. *Hepatology*, 2012. 56(3): p. 1004-14.
- [14]. Richichi, C., et al. , Marker-independent method for isolating slow-dividing cancer stem cells in human glioblastoma. *Neoplasia*, 2013. 15(7): p. 840-7.
- [15]. Wang, W.J., et al. , MYC regulation of CHK1 and CHK2 promotes radioresistance in a stem cell-like population of nasopharyngeal carcinoma cells. *Cancer Res*, 2013. 73(3): p. 1219-31.
- [16]. Weng, D.S., et al. , Minimally invasive treatment combined with cytokine-induced killer cells therapy lower the short-term recurrence rates of hepatocellular carcinomas. *J Immunother*, 2008. 31(1): p. 63-71.
- [17]. Pan, K., et al. , The efficacy of cytokine-induced killer cell infusion as an

adjuvant therapy for postoperative hepatocellular carcinoma patients. *Ann Surg Oncol*, 2013. 20(13): p. 4305-11.

[18]. Contag, C.H., et al. , Definition of an enhanced immune cell therapy in mice that can target stem-like lymphoma cells. *Cancer Res*, 2010. 70(23): p. 9837-45.

[19]. Gammaitoni, L., et al. , Effective activity of cytokine-induced killer cells against autologous metastatic melanoma including cells with stemness features. *Clin Cancer Res*, 2013. 19(16): p. 4347-58.

[20]. Sangiolo, D., et al. , Cytokine-induced killer cells eradicate bone and soft-tissue sarcomas. *Cancer Res*, 2014. 74(1): p. 119-29.

[21]. Orford, K.W. and D.T. Scadden, Deconstructing stem cell self-renewal: genetic insights into cell-cycle regulation. *Nat Rev Genet*, 2008. 9(2): p. 115-28.

[22]. Sang, L., H.A. Collier, and J.M. Roberts, Control of the reversibility of cellular quiescence by the transcriptional repressor HES1. *Science*, 2008. 321(5892): p. 1095-100.

[23]. Pece, S., et al. , Biological and molecular heterogeneity of breast cancers correlates with their cancer stem cell content. *Cell*, 2010. 140(1): p. 62-73.

[24]. Kusumbe, A.P. and S.A. Bapat, Cancer stem cells and aneuploid populations within developing tumors are the major determinants of tumor dormancy. *Cancer Res*, 2009. 69(24): p. 9245-53.