

Original Article

Fluctuation and significance of circulating endothelial cells during the therapeutic process of transarterial chemoembolization of hepatocellular carcinoma

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Abstract: Purpose: To investigate the predictive value of circulating endothelial cells (CECs) to evaluate the efficacy of transarterial chemoembolization (TACE) and their potential value to guide selection of the time interval of TACE. Materials and methods: 35 hepatocellular carcinoma (HCC) patients suitable for TACE and 10 healthy volunteers were enrolled in this study. Three milliliters of peripheral blood of patients were drawn 1 day before TACE and 3 days, 7 days, 14 days and 30 days after TACE respectively for assay of CECs. The basic level of controls and patients were compared. According to different clinical features such as age, sex, Child-pugh score, AFP, BCLC staging, the patients were classified into different groups. And the basic level of CECs of different groups was compared. Then we compared the level of CECs of 5 time points during the treatment period of TACE. 30 days after TACE we evaluated the efficacy of treatment using mRECIST criteria and analysed the relationship between level of CECs and clinical outcome. The relationship between AFP and CECs was also analysed. Results: The mean basic CECs level of patients was significantly higher than that of the control group (14.06 ± 7.28 vs. 4.70 ± 2.36 , $P=0.000$). In comparisons between different groups according to the patients' clinical features, the significant difference of the mean CECs levels was only found between Child-pugh A and B groups (12.52 ± 5.51 vs. 19.25 ± 10.25 , $P=0.019$). The study exhibited a fluctuating decreasing tendency of CEC levels during effective therapies. The baseline level of CECs in good therapeutic effect and poor therapeutic effect group has no significant difference (15.05 ± 7.74 vs. 12.88 ± 6.77 , $P=0.381$). While that of day 30 in good therapeutic effect group was significantly lower than poor therapeutic effect group (6.68 ± 3.87 vs. 10.81 ± 5.79 , $P=0.022$). The basic and day 30 levels of CECs and AFP had both significant positive correlation (Pearson correlation coefficient = 0.409, $P=0.015$; Pearson correlation coefficient = 0.381, $P=0.024$). Conclusion: Our study suggests that the trend of CECs variations during therapeutic process could be an ideal marker to evaluate the efficacy of TACE and choose the optimal time point to carry out TACE.

Keywords: Hepatocellular carcinoma, circulating endothelial cell, transarterial chemoembolization

Introduction

Hepatocellular carcinoma (HCC) is one of the most aggressive solid tumors associated with poor prognosis [1]. Wider application of screening program for high-risk populations has led to increasing detection of early tumors amenable to curative surgical treatment [2]. However, most patients present with symptomatic disease, often with bilobar tumors, portal vein invasion, or metastasis, and are therefore incurable by surgery [3]. Nonsurgical strategies such as transarterial chemoembolization (TACE) are the palliative treatment options [1].

Little is known of the tumor biology of HCC that leads to its rapid growth and metastasis. One of the striking characteristics of HCC is its rich tumor neovascularization, which can be clearly observed in various medical imaging modalities [4]. Angiogenesis is essential for the development, progression, and invasion of solid tumors. Tumor neovascularization requires recruitment and proliferation of endothelial cells [5].

Circulating endothelial cells (CECs) are usually perceived as markers that indicate the formation of new microvessels when small vessels are injured. The CEC levels of patients with carci-

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noma are significantly higher than those in healthy volunteers, suggesting that CECs are involved in angiogenesis induced by malignancies that provide tumor vasculature. CECs can exert vascular formation [6]. CECs are positively correlated to VEGF in serum and descend to normal range after resection of tumor or chemotherapy [7, 8]. Thus, CECs could be considered potential ideal indicators of anti-angiogenic therapeutic efficacy.

TACE is the standard therapy for HCC patients who are not suitable for surgical treatment. TACE concentrates on chemotherapeutic agents at the tumor site while blocking the primary artery from feeding the tumor. Thus, TACE is widely used to prolong the survival of patients with HCC. However, this procedure can stimulate local angiogenic factors that facilitate tumor regrowth and increase the possibility of metastasis [9]. Meanwhile HCC patients usually took a series of TACEs to control the lesion repeatedly. But there is no objective standard to decide the time interval of TACE yet. It is urgent to find a potential biomarker to evaluate the efficacy and prognosis of TACE and to guide to select the optimal time interval to repeat TACE.

In the present study, we explored the variation of CECs level during the process of TACE treatment and to investigate their predictive value for efficacy of TACE and their potential value to guide selection of the time interval of TACE.

Materials and methods

Patients

We prospectively recruited 35 patients with HCC and full clinical data from August 2012 to August 2014. Patients with HCC were included if they met the diagnostic criteria outlined in Diagnostic and Treatment Practices for Hepatocellular Carcinoma (2011 edition, People's Republic of China) and had an indication for TACE, had not been treated with radiochemotherapy or other antitumor therapies, had measurable lesions and no radiographic evidence of distant metastasis, and volunteered to participate and undergo regular follow-up. Exclusion criteria includes rupture of a liver tumor and significant shunt of the hepatic artery-portal vein or hepatic artery-hepatic vein, lack of blood supply to the tumor, widespread metastatic tumor with estimated survival of less

than 3 months, cachexia or multiple organ failure, liver function of Child-Pugh level C, occlusion of the second hepatic hilum or inferior vena cava, severe anemia. 10 healthy volunteers were also included as controls. The study was approved by the ethics committee of Qilu Hospital of Shandong University. Informed consents were obtained from the participants.

TACE

All the procedures were taken using Philips FD20 or Siemens Artis Z digital angiographic system. 5F RF catheter was selectively catheterized into superior mesenteric artery, celiac trunk and common hepatic artery to make an angiography. 3F microcatheter was superselectively catheterized into branches of hepatic artery to make it clear the location, size and supply artery of the tumor. The chemotherapy protocol we used was duromycin (30-50 mg), lipoplatin (50 mg) or auxplatin (100-150 mg), 5-FU (0.75-1.0 g) and calcium folinate (0.3-0.4 g). The embolization materials were lipidol (8-20 ml) and gelfoam particles. 5-FU and calcium folinate were infused into the tumor, while duromycin, platin drugs and lipidol were mixed together to embolize the feeding arteries. After embolization, angiography was made to evaluate the effectiveness of the procedure. Then the catheter and sheath were withdrawn and pressed bandage was done. After the procedure, hydration and protection of liver function were carried out for 3-5 days.

Blood collection

Three milliliters of peripheral blood of patients were drawn 1 day before TACE and 3 days, 7 days, 14 days and 30 days after TACE respectively. All blood samples were anticoagulated with EDTA and stored at 4°C before use. Blood samples of healthy volunteers were obtained from regular physical examination center of our hospital.

Assay for CECs

Human PBMC were stained with fluorescein isothiocyanate (FITC)-conjugated anti-CD45 mAb, R-phycoerythrin (PE)-conjugated anti-CD146 mAb and allophycocyanin (APC)-conjugated anti-CD31 mAb and incubated in the dark at room temperature for 30 min. After red cell lysis with lysing solution (BD Biosciences), the samples were analyzed by FACS Canto II flow

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Table 1. Baseline characteristics of 35 patients

Variables	Data
Sex (male/female)	30 (85.7%)/5 (14.3%)
Age (years)	58.6 (29-79)
Hepatitis virus background (HBV/HCV)	33 (94.3%)/2 (5.7%)
Child-pugh score (A/B)	27 (77.1%)/8 (22.9%)
AFP (>400 ng/ml/<400 ng/ml)	29 (82.9%)/6 (17.1%)
BCLC (B/C)	26 (74.4%)/9 (25.6%)
Tumor burden	
Diameter of the largest tumor \geq 5 cm	15 (42.9%)
Number of nodules >3	13 (37.1%)
Lobar distribution (unilobar/bilobar)	25 (71.4%)/10 (28.6%)

SD or PD is considered as poor therapeutic effect.

Statistical analysis

All analyses were performed using statistical software SPSS19.0. Results were expressed as mean \pm SD. CECs levels between groups were compared using t-test. Spearman's correlation analysis was performed to investigate the correlation between CECs count and AFP. Differences were considered statistically significant at $P < 0.05$ on two-tailed test.

Table 2. Comparison of basic level of CECs between groups of different clinical features

Clinical features	CECs (/500,000 cells)	P value
Age		
\geq 60	12.95 \pm 6.65	0.976
<60	15.67 \pm 8.07	
Sex		
Male	13.73 \pm 7.74	0.626
Female	16.00 \pm 3.46	
Child-pugh score		
A	12.52 \pm 5.51	0.019
B	19.25 \pm 10.25	
AFP		
>400 ng/ml	14.76 \pm 7.61	0.103
<400 ng/ml	10.67 \pm 4.50	
BCLC		
B	13.38 \pm 7.65	0.315
C	16.00 \pm 6.10	

Results

Patient's characteristics

A total of 35 patients and 10 healthy controls were enrolled in the present study. Characteristics of patients are summarized in **Table 1**. The majority of the patients were men (85.7%); the median age was 58.6 years. The most common cause of HCC is hepatitis B virus (94.3%). Liver function of 27 patients (77.1%) was Child-Pugh class A. 9 patients (25.6%) had portal vein thrombosis. 15 patients (42.9%) had a largest nodule diameter \geq 5 cm and 13 (37.1%) had more than 3 tumor nodules. Most patients (82.9%) had an Alpha-Fetoprotein (AFP) level which more than 400 ng/mL.

The basic level of CECs in patients and control group

The mean CECs level of Patients before TACE was 14.06 \pm 7.28, which was significantly higher than that of the control group (4.70 \pm 2.36, $P = 0.000$). According to the patients' clinical features, such as age, sex, Child-pugh score, AFP, BCLC staging, they were classified into different groups. And the mean CECs levels of different groups were compared. The significant difference was only found between Child-pugh A and B groups (12.52 \pm 5.51 vs. 19.25 \pm 10.25, $P = 0.019$). Details were shown in **Table 2**.

Variation of CECs level during the therapeutic period of TACE

There was a remarkable fluctuation of CECs during the therapeutic process of TACE (**Figure 1**). 3 days after TACE, the CECs level of patients decreased from 14.06 \pm 7.28 to 13.91 \pm 6.61, but there was no significant difference ($P = 0.856$). 7 days after TACE, the CECs level of

cytometer (BD Biosciences, San Jose, CA, USA) for the detection of CECs (CD45⁺CD146⁺CD31⁺). In total, 500,000 blood cells were analyzed and cell count of the identified cell population is given in relation to the 500,000 measured blood cells. Isotype controls, PE-conjugated mouse IgG2a, FITC and APC-conjugated mouse IgG1, κ mAb were used. All antibodies used here were purchased from Biologend (San Diego, CA, USA).

Evaluation of efficacy

CT or MR examinations were performed before and 30 days after TACE. Efficacy was classified into complete response (CR), partial response (PR), stable disease (SD), and progressive disease (PD) according to the mRECIST criteria. CR or PR is considered as good therapeutic effect.

Fluctuation of CECS during TACE

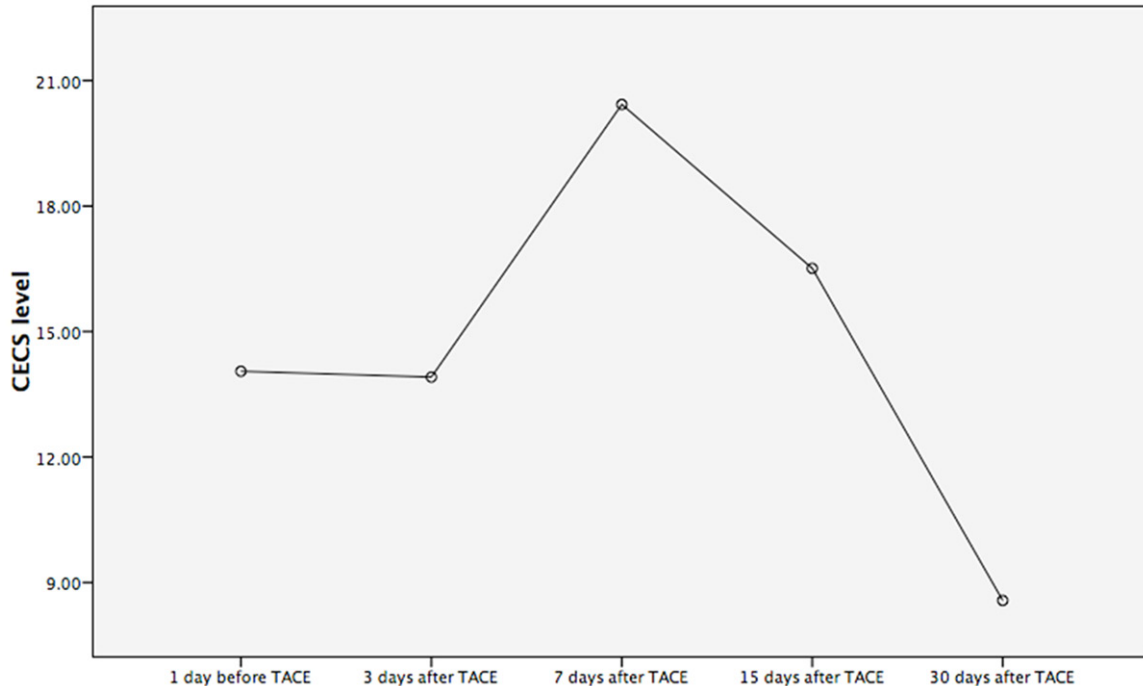


Figure 1. Fluctuation of CECS level during the TACE therapy process. There is a remarkable increasing on day 7 and decreasing on day 30, compared to the baseline level.

patients increased from 14.06 ± 7.28 to 20.43 ± 9.61 significantly ($P=0.000$). 14 days after TACE, the CECS level (16.51 ± 6.34) also showed a significant increase compared to the baseline level ($P=0.049$). But compared to that of day 7, a significant decreasing had been shown ($P=0.003$). 30 days after TACE, the CECS level had decreased to 8.57 ± 5.20 , which was significantly decreased compared to baseline, day 7 and day 14 ($P=0.000$).

CECs count: correlation with clinical outcome

Out of 35 patients, 2 CR, 17 PR, 12 SD and 4 PD were found 1 month after TACE according to mRECIST criteria. The baseline level of CECS in CR/PR and SD/PD group has no significant difference (15.05 ± 7.74 vs. 12.88 ± 6.77 , $P=0.381$). 30 days after TACE the CECS level of CR/PR group (6.68 ± 3.87) decreased significantly compared to the baseline level ($P=0.000$). While that of SD/PD group (10.81 ± 5.79) also decreased compare to the baseline level, but there was no significant difference ($P=0.122$). And the CECS level of day 30 in CR/PR group was significantly lower that of SD/PD ($P=0.022$).

Correlation between CECS and AFP

The baseline level of CECS and AFP in the 35 patients both decreased significantly 30 days

after TACE ($P=0.000$). The baseline level of CECS and AFP had significant positive correlation (Pearson correlation coefficient = 0.409, $P=0.015$). And the day 30 level of CECS and AFP had significant positive correlation too (Pearson correlation coefficient = 0.381, $P=0.024$) (Figures 2, 3).

Discussion

Transarterial chemoembolization remains as the main therapy for unresectable HCC. Although the efficacy is encouraging, there are still some issues that need to be explored. For example, there is still no reliable biomarker to predict the efficacy and prognosis of TACE. And usually a HCC patient needs receive several TACE procedures to control the lesion. But how to choose the optimal time to carry out TACE lacks objective criteria.

Nowadays AFP is a popular predictor to monitor the patients' response to TACE in clinical application [10]. Decrease of AFP is usually presented at the one-month follow-up. But there are about 30% AFP negative HCC patients that need other predictor to evaluate the outcome of therapy. Meanwhile even a dramatically decrease of AFP was found in the AFP positive patients, only a few could drop to the normal range. If the patients whose AFP is still above

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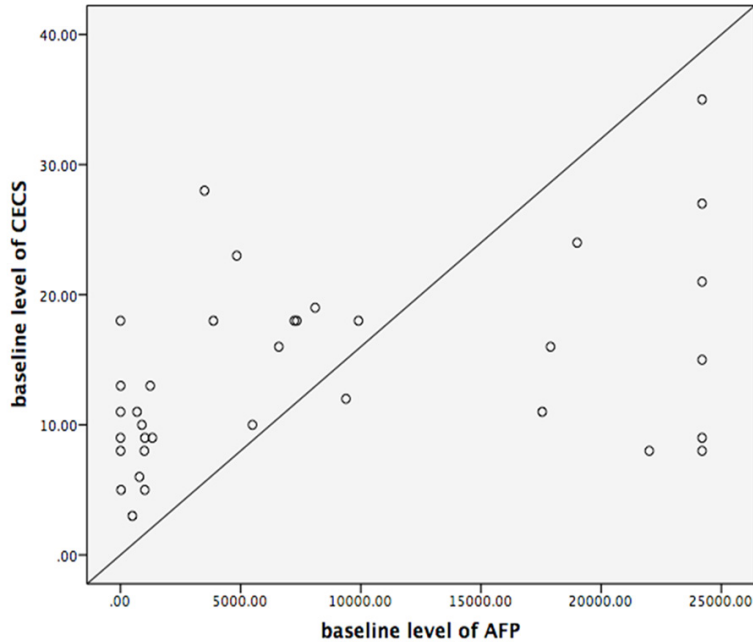


Figure 2. The baseline level of CECS and AFP showed a significant positive correlation.

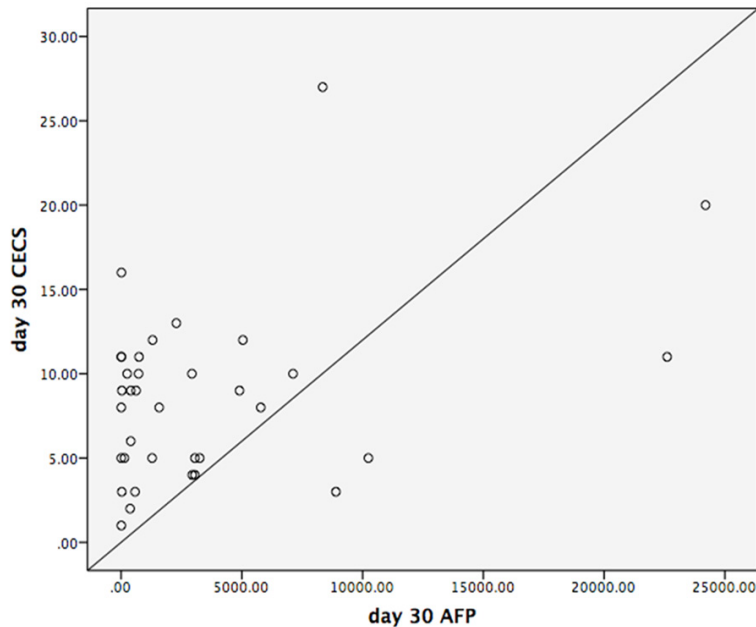


Figure 3. The level of CECS and AFP on day 30 also showed a significant positive correlation.

the upper limit need to receive another TACE is usually empirical. And imaging and clinical representation need to be taken into consideration to determine further therapeutic regimen.

In the present study, we found that the level of CECS and AFP had positive correlation either at

the baseline or on the day 30, which means CECS has the potential to be a predictor. Because of the limited patients, the cutoff value or normal range of CECS could not be established. But the CECS level of day 30 in some patients had really dropped to the level that is similar to the control group, although their AFP was still high. In this study, 4 AFP negative patients were enrolled, including 1 CR, 2 SD and 1 PD. The day 30 CECS level of the CR patient decreased remarkably from the baseline (11/ μ l to 1/ μ l). While the day 30 CECS level of the PD patient increased from baseline 9/ μ l to 11/ μ l, which showed CECS level has the potential to be used to evaluate the response to TACE in AFP negative patients.

It suggests that CECS exhibit general reducing tendency with fluctuation when therapy is effective, in which intermittent elevation means diminution of tumor vasculature by normalization, while final reduction reflects apoptosis of CECS, decrease of TAFs, and regression of vasculature in tumor. To test the hypothesis above, we investigated the change of CECS at 5 time points during 30 days. A fluctuation was really found. At day 3, a slight decrease showed. At day 7 the CECS level increased significantly compared to baseline level. Then the CECS level began to decrease. Until day 30, the CECS level had dropped dramatically to a level that had significant difference compared to baseline level. Our result is similar to the results of Beaudry et al. [11] and Liu et al. [12], which suggests the fluctuation of CECS during treatment may indicate the moving balance of vessel normalization and CEC apoptosis.

We perceive the possible mechanisms that cause the increment of CECs are as the followings. Firstly, TACE could cause the diminution of tumor vessels. The diminution of the tumor vasculature area after embolization could induce the shedding of endothelial cells from the blood vessel walls to augment CEC population. Secondly, the increase in the CECs could be due to the mobilization of endothelial progenitor cells (EPCs) from the bone marrow induced by increasing tumor angiogenesis factors (TAFs), such as vascular endothelial growth factor (VEGF), b-fibroblast growth factor, and platelet-derived growth factor, which will increase after TACE. TACE acts by inducing tumor ischemia and necrosis through arterial embolization in addition to its anticancer effect by regional chemotherapy. Hypoxia-induced angiogenesis is an important mechanism in the tumor growth induced by ischemia, and VEGF is a key mediator of this process. Some recent studies have provided evidence that VEGF mediates hypoxia-stimulated angiogenesis in HCC patients after TACE temporally [13, 14].

The potential value of pre-treatment CECs count in predicting the clinical outcome is still debated in cancer patients regarding the method used to enumerate CECs. Data suggest that CECs may be useful for predicting clinical response to chemotherapy or prolonged disease stabilization in breast cancer [15-17]. Kawaishi et al. reported that higher baseline CECs values were observed in NSCLC patients with PR/SD than those with PD after one cycle of carboplatin and paclitaxel [18]. In our study, baseline CECs levels between CR/PR and SD/PD group has no significant difference, which may be due to the fact that CECs could be influenced by various factors related to angiogenesis, tumor vasculature, and tumor localization. While 30 days after TACE the CECs level of CR/PR group decreased significantly compared to the baseline level. That of SD/PD group also decreased compare to the baseline level, but there was no significant difference. And the CECs level of day 30 in CR/PR group was significantly lower that of SD/PD. These findings suggest it is the Δ CECs (difference between baseline level of CECs and that of 30 days after TACE), not the baseline level of CECs that could be used as a predictive marker for response to TACE in HCC.

Our results exhibited a fluctuating decreasing tendency of CEC levels during effective thera-

pies, which could ascended in some time points during therapy, but ultimately descended and maintained at a low level. Our study suggests that the trend of CECs variations between pre- and post-therapies could be an ideal marker for the efficacy of TACE and choosing the optimal time point to carry out TACE. According to the AFP negative patients, CECs could be even more helpful to guide therapy. If Nowadays the combined TACE and anti-angiogenic therapy has been showing challenging outcome. CECs would also be suitable to monitor the anti-angiogenic effect to guide the treatment regimen. Larger clinical trials are needed to confirm these conclusions and explore the usefulness of CECs in comprehensive therapy in HCC patients.

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Disclosure of conflict of interest

None.

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