Impact of liver tumor percutaneous radiofrequency ablation on circulating tumor cells

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Abstract. Radiofrequency ablation has become an increasingly common therapeutic technique for patients with hepatocellular carcinoma or metastatic liver tumors. However, reports on the effect of percutaneous radiofrequency ablation (PRFA) on circulating tumor cells (CTCs) are limited. The present study aimed to further investigate the impacts of PRFA on the numbers and phenotypes of CTCs in patients with hepatocellular carcinoma or metastatic liver tumors. A total of 43 patients with hepatocellular carcinoma or 7 types of metastatic liver tumors were treated with PRFA. A total of 5 ml blood per sample were collected from the peripheral circulation 30 min before and 3 days after PRFA. The total number of CTCs significantly increased 3 days after PRFA, and the mesenchymal phenotype CTCs, which also increased significantly, significantly contributed to the overall increase in CTCs. Furthermore, the lymphocyte levels were significantly decreased following PRFA, and the CTC level was significantly higher in patients with decreased lymphocyte levels compared with those with increased lymphocyte levels. Liver tumor PRFA may increase the level of mesenchymal phenotype CTCs, which is significantly associated with the lymphocyte count. Factors pertaining to the performance of PRFA were also investigated in the present research, but no significant results were identified.

Introduction

Hepatic blood flow is abundant due to the hepatic artery and portal vein double blood supply. Cancer cells originating from systemic organs are able to metastasize to the liver through the circulatory system. In general, ~50% of patients with colorectal cancer develop liver metastases, and 80-90% of these liver metastases are contraindicated for radical resection (1-4). Liver metastasis is also a significant factor contributing to the high mortality rate in patients with colorectal cancer (5). The median survival time of patients with untreated liver metastases was 6.9 months, and the 5-year survival rate of the patients with unresectable liver metastases was close to 0% (6,7).

Liver metastases seriously influence patient survival. Percutaneous radiofrequency ablation (PRFA) converts radiofrequency waves into heat energy, which leads to tissue dehydration and eventually results in the coagulative necrosis of cells. This efficacious technique has been used for >20 years, and evidence has demonstrated that the short-term and long-term efficacies of RFA have no statistical difference compared with that of surgery in hepatocellular carcinoma with a tumor diameter of <5 cm (8). As for colorectal liver metastases, research has revealed that although the rate of recurrence-free survival in RFA is lower compared with that in resection, the 5-year overall survival rate is as high as 48%, which is similar to that of surgical resection (9,10). Compared with surgery, open RFA and PRFA both demonstrated to increase the number of circulating tumor cells (CTCs) in peripheral circulation (11). However, such reports included a small number of patients with hepatocellular carcinoma and/or colorectal liver metastases, and did not the explore changes in the phenotypes of CTC and other associated factors. The present study focuses on liver metastases of multiple cancer types. To the best of our knowledge, the present study has demonstrated, for the first time, the association between factors in the performance of PRFA and changes in the CTC level, including changes in CTC phenotype.

CTCs are tumor cells in the circulatory system that originate from solid tumor lesions, including hepatocellular carcinoma lesions and metastatic tumor lesions. They enter the circulatory system as a result of diagnostic or therapeutic surgery, or spontaneous tumor shedding. The majority of CTCs are subjected to apoptosis or phagocytosis in the circulatory system, and only a few survive and develop into metastatic lesions due to the unique microenvironment in the circulating system. CTCs are able to significantly improve the rate of patient mortality. Various reports confirmed that CTCs are a significant prognostic indicator (12,13) and are even more accurate compared with imaging modalities to a certain extent (14). The changes in the numbers and phenotypes of CTCs reflect

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the tumor status and effect of treatment in real time. Methods for detecting CTCs have significantly approved in the recent years, including immune-magnetic separation (15) and quantitative polymerase chain reaction-based assays (16). In the present study, CTCs were examined using the second-generation CanPatrol CTCs detection technology (17), which allows for the monitoring of changes in the numbers and phenotypes, including the epithelial phenotype, mesenchymal phenotype and mixed epithelial/mesenchymal phenotype, of CTCs.

The present study focused on the impact of liver tumor PRFA on the quantity of CTCs, and also the CTC phenotypes and other blood parameters in patients with different tumors, including the following: Colorectal cancer liver metastases, hepatocellular carcinoma, gastric cancer liver metastases, nasopharyngeal carcinoma liver metastases, small intestine cancer liver metastases, ovarian cancer liver metastases, breast ductal carcinoma liver metastases, and ampullary carcinoma liver metastases; and treatment backgrounds (chemotherapy and targeted therapy). Changes in the quantity and phenotypes of CTCs, blood count, and liver function indexes were evaluated in total patients, while immune cell subsets and tumor markers were evaluated in only a portion of the patient population. Given that RFA has been reported to affect the immune system (18,19), lymphocyte subsets were also evaluated the current study. As for PRFA surgery, the target tumor burden, number of ablation points, puncture times, treatment time and ablation location (liver left lobe and/or right lobe) were included in the analysis. The target tumor burden was calculated as the sum of the longest diameter of the target tumor. The impact of liver tumor PRFA on CTCs and other blood indexes in patients with different backgrounds was comprehensively assessed.

Patients and methods

Patient characteristics. Between May 2016 and August 2017, 43 patients (28 male; 15 female) with a mean age of 53 years (range, 22-73 years) who had been diagnosed with any type of malignant liver tumor, including hepatocellular carcinoma and liver metastases, via definite pathological and imaging modalities were recruited. The present study was approved by the medical ethics committee of Nanfang Hospital, Southern Medical University (Guangzhou, China), and written informed consent was obtained from all patients. After all patients underwent comprehensive enhanced computed tomography (CT) or magnetic resonance imaging (MRI) and multidisciplinary treatment, no patient in the current study was identified to be eligible for surgical resection of liver metastases. Consequently, individualized RFA plans were created based on the status of the liver tumors that were evaluated using the last enhanced CT or MRI.

Flow cytometric analysis of human blood cells. Routine blood test (complete blood count) and liver function indexes were assessed in all patients 30 min before and 3 days after PRFA. The total number and classification count of leukocytes were measured by flow cytometry in a Sysmex XE2100 automatic blood analyzer (Sysmex Corporation, Kobe, Japan). In brief, blood samples (200 μl blood per sample) were mixed with Stromatolyser-4DL (Sysmex Corporation) and treated with Stromatolyser-4DS (Sysmex Corporation) at 41˚C for 22 sec, containing 0.002% polymethine dye to stain the nucleus and organelles. Then, the samples were irradiated with a semiconductor laser, whereby the dye produces different intensities of fluorescence. Each cell detected three different scattering angles: Forward scattered light (representing cell volume), lateral scattered light (representing contents of a cell, including nucleus or granules) and lateral fluorescence (representing contents of DNA and RNA). Using this automatic blood analyzer and flow cytometry method, monocyte and lymphocyte counts were obtained. Furthermore, studies using the same automatic blood analyzer and method have also been published previously (20-22). In addition, immune cell subsets and tumor markers were assessed in certain patients 30 min before and 3 days after PRFA. Immune cell subsets were detected by flow cytometry. In brief, 50 μl anticoagulant whole blood sample was incubated for 20 min at room temperature with 20 μl of the following labelled antibodies: Cluster of differentiation (CD)4-fluorescein isothiocyanate (FITC)/CD8-PE/CD3-PerCP (cat no. 340298; BD Biosciences, Franklin Lakes, NJ, USA), IOTEST CD3-FITC/CD (16+56)-PE (cat no. 340300; Beckman Coulter, Inc., Brea, CA, USA). Then, 300 μl hemolysin (BD Biosciences) was added to the test tube, and incubated for 20 min at room temperature for full hemolysis to occur. Next, 1 ml sheath (Jinan Xisenmeikang Medical Electronic Co., Ltd., Jinan, China) was added and the mixture was centrifuged at 180 x g for 5 min at 4˚C. After discarding the supernatant and adding 500 μl sheath into the tube, flow cytometry was performed using a FACScalibur (BD Biosciences) to detect different tumor markers (23-25), including carcinoembryonic antigen (cat no. 401-10; CanAg Diagnostics Co., Ltd., Beijing, China), CA19-9 (cat no. 120-10; CanAg Diagnostics Co., Ltd.), CA72-4 (cat no. EIA-5071; DRG Diagnostics GmbH; Marburg, Germany), CA24-2 (cat no. 101-10; CanAg Diagnostics Co., Ltd.) and α-fetoprotein (cat no. 600-10; CanAg Diagnostics Co., Ltd.) using the corresponding tumor marker kits (CanAg Diagnostics, Co., Ltd., Goteborg, Sweden).

PRFA treatment. In the present study, all patients had signed informed consent prior to PRFA treatment. All patients received PRFA treatment under conscious sedation with pethidine and local anesthesia with 2% lidocaine. The procedures were performed under ultrasound guidance. The mean PRFA treatment time was 26.6 min (range, 2.5-66.1 min).

CTC isolation and classification. Blood samples of 5 ml each were collected from the peripheral circulation 30 min before and 3 days after PRFA. The CanPatrol™ CTC enrichment technique was used to isolate and classify the CTCs as previously described (17). In brief, the CTCs were first isolated by size using a filter-based equipment comprising a filtration tube (Surexam, Guangzhou, China) with calibrated membranes with 8-μm-diameter pores (EMD Millipore, Billerica, MA, USA), manifold vacuum plate with valve setting (Surexam), an E-Z96 vacuum manifold (Omega Bio-Tek, Inc., Norcross, GA, USA), and a vacuum pump. Tri-color RNA *in situ* hybridization (RNA-ISH), which is based on the branched DNA signal amplification technology, was then performed to classify the different phenotype of CTCs according to epithelial-mesenchymal transition (EMT) biomarkers. After treating with a protease (Qiagen GmbH, Hilden, Germany) at 25˚C for 1 h, the cells on the membrane were hybridized with capture probes (Invitrogen;

Table I. Probe sequences for the EpCAM, CK8/18/19, vimentin, twist and CD45 genes.

Gene	Sequences (5'→3')	Gene	Sequences (5'→3')
EpCAM	TGGTGCTCGTTGATGAGTCA	Vimentin	GAGCGAGAGTGGCAGAGGAC
	AGCCAGCTTTGAGCAAATGA		CTTTGTCGTTGGTTAGCTGG
	AAAGCCCATCATTGTTCTGG		CATATTGCTGACGTACGTCA
	CTCTCATCGCAGTCAGGATC		GAGCGCCCCTAAGTTTTTAA
	TCCTTGTCTGTTCTTCTGAC		AAGATTGCAGGGTGTTTTCG
	CTCAGAGCAGGTTATTTCAG		GGCCAATAGTGTCTTGGTAG
CK8	CGTACCTTGTCTATGAAGGA	Twist	ACAATGACATCTAGGTCTCC
	ACTTGGTCTCCAGCATCTTG		CTGGTAGAGGAAGTCGATGT
	CCTAAGGTTGTTGATGTAGC		CAACTGTTCAGACTTCTATC
	CTGAGGAAGTTGATCTCGTC		CCTCTTGAGAATGCATGCAT
	CAGATGTGTCCGAGATCTGG		TTTCAGTGGCTGATTGGCAC
	TGACCTCAGCAATGATGCTG		TTACCATGGGTCCTCAATAA
CK18	AGAAAGGACAGGACTCAGGC	CD45	TCGCAATTCTTATGCGACTC
	GAGTGGTGAAGCTCATGCTG		TGTCATGGAGACAGTCATGT
	TCAGGTCCTCGATGATCTTG		GTATTTCCAGCTTCAACTTC
	CAATCTGCAGAACGATGCGG		CCATCAATATAGCTGGCATT
	AAGTCATCAGCAGCAAGACG		TTGTGCAGCAATGTATTTCC
	CTGCAGTCGTGTGATATTGG		TACTTGAACCATCAGGCATC
CK19	CTGTAGGAAGTCATGGCGAG		
	AAGTCATCTGCAGCCAGACG		
	CTGTTCCGTCTCAAACTTGG		
	TTCTTCTTCAGGTAGGCCAG		
	CTCAGCGTACTGATTTCCTC		
	GTGAACCAGGCTTCAGCATC		

CD45, cluster of differentiation 45. All sequences were synthesized by Invitrogen (Thermo Fisher Scientific, Inc.). The content of Table I is referenced from (17).

Thermo Fisher Scientific, Inc., Waltham, MA, USA) specific for the epithelial biomarkers EpCAM and CK 8/18/19, two mesenchymal biomarkers vimentin and twist, and the leukocyte biomarker CD45 (Table I). Then, 4',6-diamidino-2-phenylindole (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) was used to stain the CTCs, and an Olympus BX53 fluorescence microscope (Olympus Corporation, Tokyo, Japan) was used for enumeration and analysis at a magnification of x100. The red and green fluorescent signal represented epithelial CTCs and mesenchymal CTCs, respectively. Mixed epithelial/mesenchymal phenotype CTCs were recognized as red and green fluorescent signal. EpCAM and CK are commonly used for epithelial CTC detection (26-28), but one report revealed that the epithelial marker-based enrichment method results in failure of CTC detection due to the downregulation of EpCAM have been published (29). The CanPatrol CTC enrichment technique classifies CTCs that do not express EpCAM, including the brain glioma cell line U118MG (17). Furthermore, papers using the CanPatrol CTC enrichment technique have also been published recently (30-32).

Statistical analysis. Paired-sample t-tests were used to analyze the significant differences between matched data. Independent-sample t-tests and one-way analysis of variance followed by least significant difference and Dunnett's post-hoc tests were performed to test the significant differences among groups of data. Bivariate correlation analysis was also used to explore the correlativity between target data and changes in the value of CTCs, followed by Spearman correlation coefficient. Data are presented as mean ± standard deviation (n=43), and $P<0.05$ was considered to indicate a statistically significant difference. All data analyses were performed using IBM SPSS Statistics 22 (IBM Corp., Armonk, NY, USA).

Results

Effect of the general background and tumor background on CTC level. Age, sex, primary tumor type, T stage, N stage, number of distant metastases, and the time interval between the diagnosis of liver tumors and the PRFA were included in the analysis. The average age of patients was 53 years (range, 22-73 years), and the male-to-female ratio was 28:15. The cohort comprised 27 cases of colorectal cancer liver metastases, 7 hepatocellular carcinoma, 3 gastric cancer liver metastases, 2 nasopharyngeal carcinoma liver metastases, 1 small intestine cancer liver metastases, 1 ovarian cancer liver metastases, 1 breast ductal carcinoma liver metastases and 1 ampullary carcinoma liver metastases (Table II). The distant metastases included liver,

Table II. Patient general background and tumor background.

Characteristic	No. of patients (%)	Mean difference in CTCs[a]	P-value[b]
Age (years)			0.940
<60	28 (65)	3.96	
≥60	15 (35)	3.73	
Sex			0.516
Male	28 (65)	4.57	
Female	15 (35)	2.6	
Tumor types			0.232
Colorectal cancer liver metastasis	27 (63)	4.12	
Hepatocellular carcinoma	7 (16)	1.71	
Gastric cancer liver metastases	3 (7)	13.33	
Nasopharyngeal carcinoma liver metastasis	2 (5)	-1	
Small intestine cancer liver metastasis	1 (2)	6	
Ovarian cancer liver metastasis	1 (2)	4	
Breast ductal carcinoma liver metastases	1 (2)	-1	
Ampullary carcinoma liver metastases	1 (2)	-1	
T stage			0.649
2	1 (2)	2	
3	9 (21)	1.89	
4	26 (61)	5.46	
N stage			0.560
0	9 (21)	4.33	
1	11 (26)	2.27	
2	12 (28)	7	

[a]Total CTC number following PRFA minus CTC number prior to PRFA. [b]Difference in total CTC number (post PRFA minus pre) divided into different groups according to each characteristic listed. P-values were obtained using an independent sample t-test or one-way analysis of variance test with post hoc test incuding LSD and Dunnett T3. PRFA, percutaneous radiofrequency ablation; CTCs, circulating tumor cells; T, tumor stage; N, node stage.

Table III. Liver tumor background.

Characteristic	No. of patients (%)	Mean difference in CTCs[a]	P-value[b]
Number of distant metastases			0.137
Liver metastasis only	18 (42)	1.67	
Liver metastasis with extrahepatic metastasis	25 (58)	5.48	
Time interval between the diagnosis of liver tumor and PRFA, months			0.313
<12	32 (74)	3.03	
≥12	11 (26)	6.36	

[a]Total CTC number following PRFA minus CTC number prior to PRFA. [b]Difference in total CTC number (post PRFA minus pre) divided into different groups according to each characteristic listed. P-values were obtained using an independent sample t-test or one-way analysis of variance test with post hoc test incuding LSD and Dunnett T3. PRFA, percutaneous radiofrequency ablation; CTCs, circulating tumor cells.

lung, bone, brain, adrenal gland, ovary and distant lymph nodes. Distant metastasis was then divided into two groups: Liver metastases only and liver metastases with extrahepatic metastases, such as distant organ and regional lymph node metastases. The time interval between the diagnosis of liver tumors and the PRFA was also divided into two groups according to the median time interval of 5.6 months. All P-values were >0.05, and the results of the analysis demonstrated that the general background and tumor background of patients did not affect the CTC level following PRFA (Tables II and III).

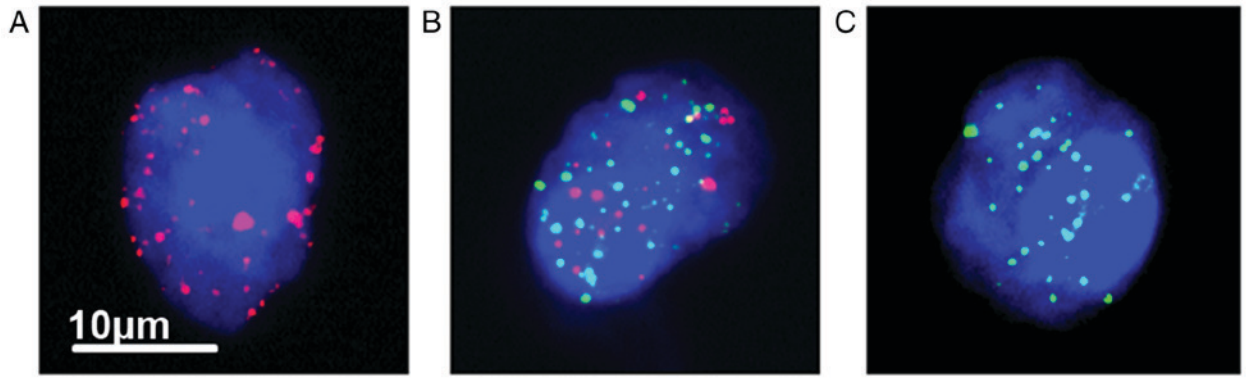

Figure 1. Images of three different phenotype CTCs under fluorescence microscope. (A) Epithelial phenotype CTCs with red fluorescent signal. (B) Mixed epithelial/mesenchymal phenotype CTCs with red and green fluorescent signal. (C) Mesenchymal phenotype CTCs with green fluorescent signal. CTCs, circulating tumor cells.

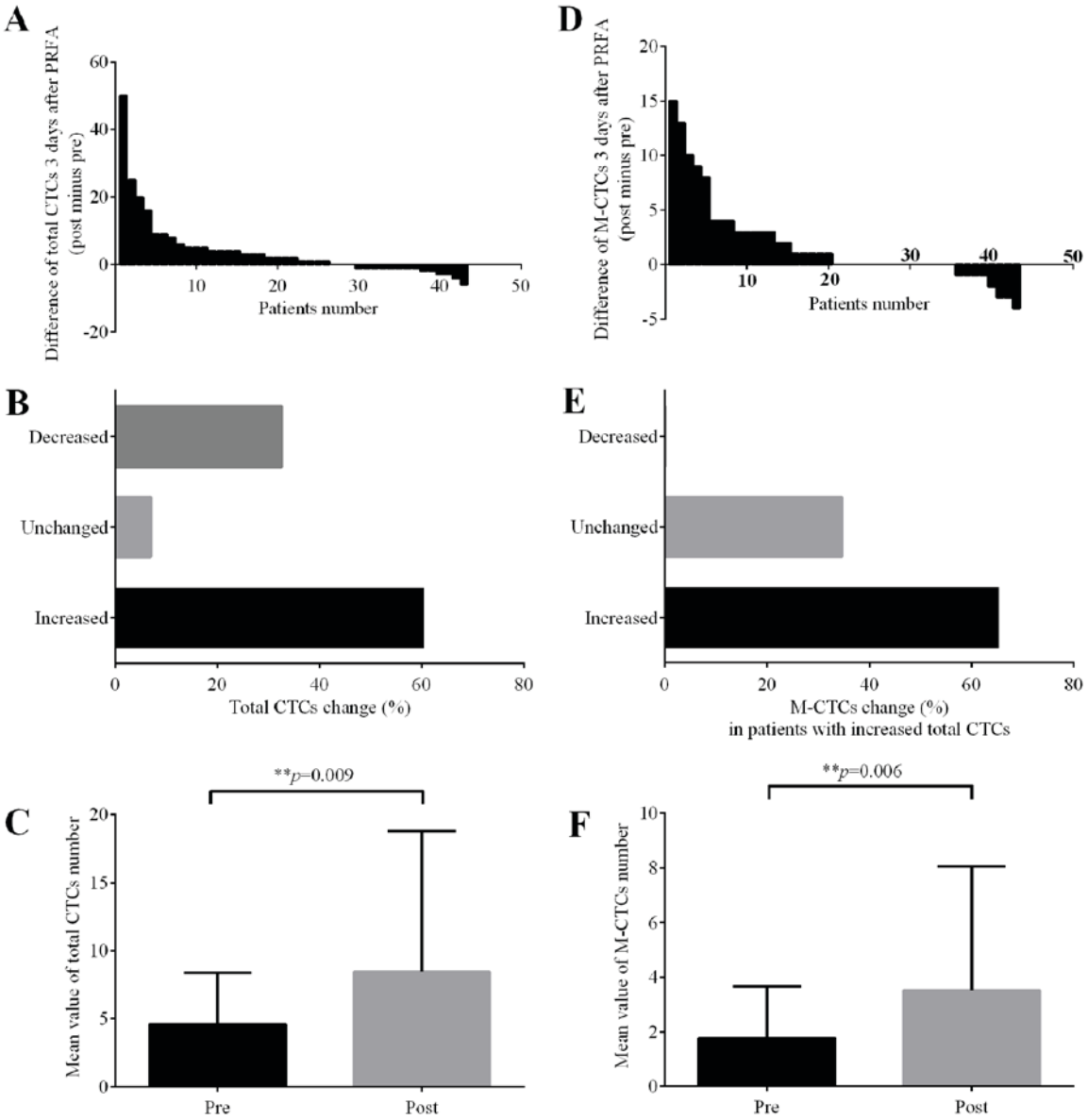

Figure 2. Changes in the total CTC level and mesenchymal phenotype CTC of patients undergoing PRFA. (A) The differences in the total number of CTCs (post PRFA minus pre). (B) The proportion of increased, unchanged and decreased levels of CTCs following PRFA. (C) Significant differences were determined according to a paired sample t-test between the pre PRFA group and post PRFA group in terms of total CTCs (P=0.009). (D) The differences in the number of mesenchymal phenotype CTCs (post PRFA minus pre). (E) The changes in the proportion of mesenchymal phenotype CTC in patients with an increased total level of CTCs. (F) The significant differences according to a paired sample t-test between the post PRFA group and pre PRFA group in terms of mesenchymal phenotype CTCs (P=0.006). CTCs, circulating tumor cells; M, mesenchymal phenotype; PRFA, percutaneous radiofrequency ablation.

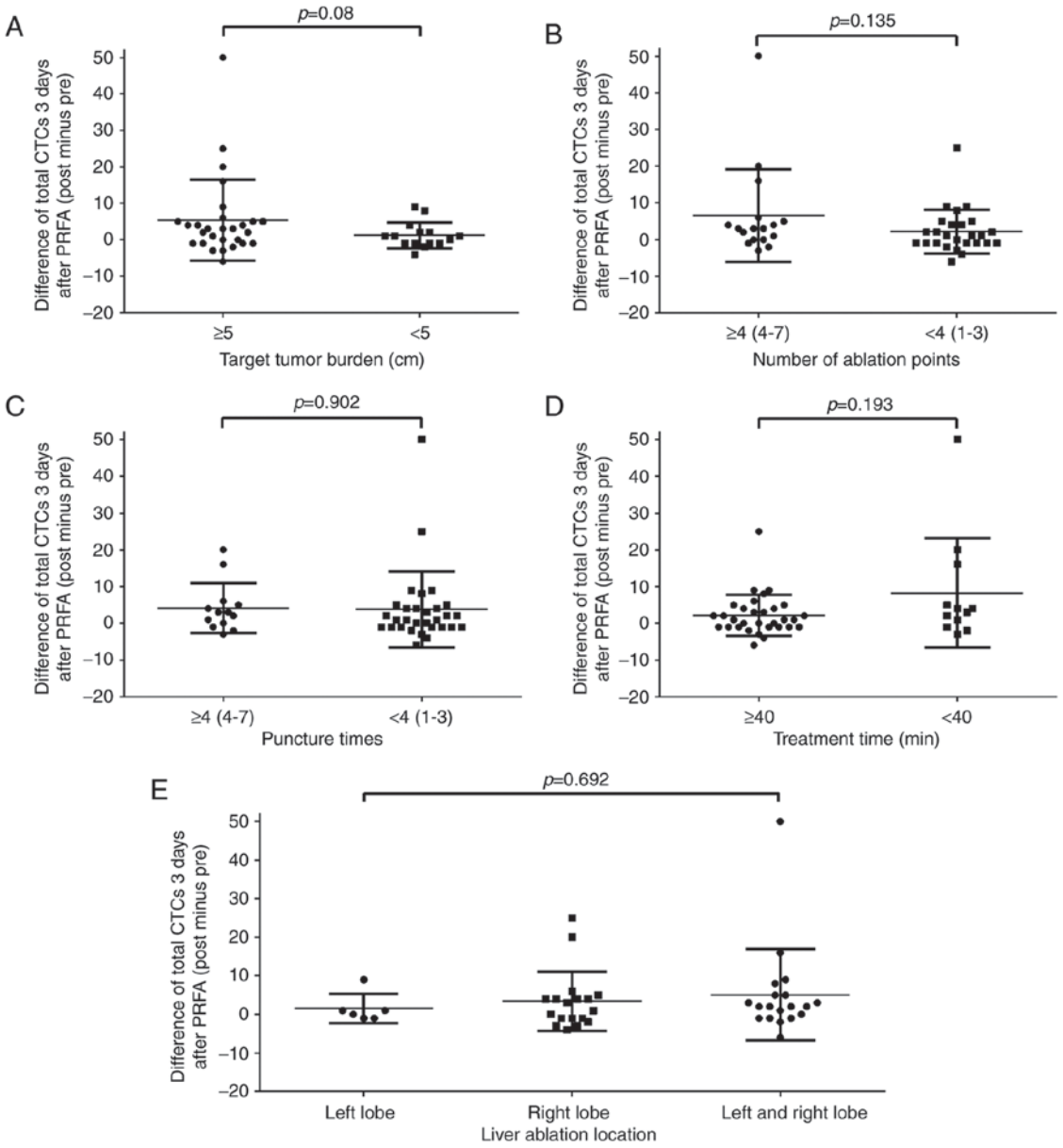

Figure 3. Effect of PRFA surgery factors on total CTC levels. (A) The difference in the total number of CTCs (post PRFA minus pre) was divided into two groups according to the target tumor burden (<5 cm group and ≥5 cm group), and no statistical difference was noted. No statistically significant difference was identified between groups according to the numbers of (B) ablation points (<4 vs. ≥4 points), (C) puncture times (<4 vs. ≥4 times), (D) treatment time (<40 vs. ≥40 min), and (E) ablation location (liver left lobe vs. right lobe vs. both left and right lobe), respectively. P-values were determined using independent sample t-test for (A-D) and one-way analysis of variance test with post hoc test including least significant difference and Dunnett's T3 post-hoc test (E). PRFA, percutaneous radiofrequency ablation; CTCs, circulating tumor cells.

Total CTC level increases following PRFA, particularly the mesenchymal phenotype CTCs. After counting the three phenotype of CTCs separately (Fig. 1), it was revealed that the CTC-positive rate prior to PRFA was 88.4% (38/43), while that 3 days after PRFA was 97.7% (42/43). Of the 43 patients with liver tumors, the CTC level increased in 26 patients (60.5%) following PRFA (Fig. 2A and B). Compared with the CTC level prior to PRFA, the CTC level 3 days after PRFA was significantly increased (P=0.009) (Fig. 2C). Meanwhile, of the 26 patients who exhibited high total CTC levels following PRFA, 17 (65.4%) had increased mesenchymal phenotype CTCs, while the number of mesenchymal phenotype CTCs remained unchanged in 9 patients (34.6%) (Fig. 2D and E). It was demonstrated that

Table IV. CTC level increase in patients who did not find CTC prior to PRFA.

Patients	CTC number prior to PRFA	CTC number following PRFA
No. 04	0	0
No. 07	0	4
No. 16	0	1
No. 17	0	25
No. 18	0	4

PRFA, percutaneous radiofrequency ablation; CTCs, circulating tumor cells.

Table V. PRFA surgery and CTC changes.

Characteristics	No. of patients (%)	Mean difference in CTCs[a]	P-value[b]	r-value[c]
Target tumor burden (cm)			0.08	0.200
≥5	28 (65)	5.32		
<5	15 (35)	1.20		
Number of ablation points			0.135	0.303
≥4 (4-7)	17 (40)	6.53		
<4 (1-3)	26 (60)	2.15		
Puncture times			0.902	0.185
≥4 (4-7)	13 (30)	4.15		
<4 (1-3)	30 (70)	3.77		
Treatment time (min)			0.193	0.218
≥40	12 (28)	8.25		
<40	31 (72)	2.19		
Ablation location			0.692	0.127
Left lobe	6 (14)	1.5		
Right lobe	18 (42)	3.39		
Left and right lobe	19 (44)	5.11		

[a]Total CTC number following PRFA minus CTC number prior to PRFA. [b]Difference in total CTC number (post PRFA minus pre) divided into different groups according to each characteristic listed. P-values were obtained using an independent sample t-test or one-way analysis of variance test with post hoc test incuding LSD and Dunnett T3. [c]Spearman correlation coefficient between the difference in total CTC number (post minus pre PRFA) and PRFA surgery factors. PRFA, percutaneous radiofrequency ablation; CTCs, circulating tumor cells.

an increasing number of mesenchymal phenotype CTCs (P=0.006) significantly contributed to the total increase in CTCs (Fig. 2F). In addition, CTC was not detected in 5 patients prior to PRFA, but 4 (80%) of them exhibited high CTC levels in the peripheral circulation 3 days after PRFA (Table IV). In addition to the CTC level, the changes in different CTC phenotypes following PRFA were evaluated. These results indicated that the level of CTCs, particularly the mesenchymal phenotype CTCs, is significantly increased following PRFA of liver tumors.

Relevance between the changes of CTCs and PRFA factors. PRFA has been revealed to increase CTC levels, but the factors contributing to such occurrences remain unknown. As such, the present study focused on the following factors in the performance of PRFA: Target tumor burden, numbers of ablation points, puncture times, treatment time and ablation location (left lobe and/or right lobe of the liver) (Fig. 3). The sum of the longest diameter of the target tumor was used as the target tumor burden. Aside from t-tests, bivariate correlation analysis was also conducted between PRFA factors and changes in the number of CTCs to determine whether the aforementioned factors affected the changes in CTC levels. However, no significant correlation was identified between changes in CTC levels and all the PRFA factors, particularly the treatment time and the number of ablation points, which were initially considered to be associated with changes in CTC levels (Table V). Notably, the number of ablation points demonstrated a partial correlation with the changes in the number of CTCs (Spearman correlation coefficient, 0.303; P=0.048). Although this correlation coefficient is not satisfactory, further studies using a larger sample size are warranted.

CTCs increases significantly in patients with reduced lymphocyte levels. To thoroughly analyze the factors that may affect the CTC level, blood indexes, including that in routine blood test and liver function indexes, were investigated in all patients, while immune cell subsets and tumor markers were evaluated in certain patients (Table VI). Tumor markers including CEA, CA19-9, CA24-2 (for colorectal cancer liver metastases patients), CA72-4 (for gastric cancer liver metastases patients) and AFP (for hepatocellular carcinoma patients). Following statistical analysis, it was determined that of the 23 patients, 12 patients exhibited elevated tumor markers (1 patient with elevated CA72-4 level, 1 patient with elevated AFP level and 10 patients with elevated CEA/CA19-9/CA24-2 levels); however, 11 patients exhibited decreased or unchanged levels of tumor markers (4 patients with decreased CEA/CA19-9/CA24-2 levels and 7 patients with unchanged CEA/CA19-9/CA24-2 levels). No significant differences were determined between the mean differences of CTCs in the two groups. Considering the possibility of bone marrow suppression as the majority of patients had received chemotherapy in the past month, changes in the levels of white blood cells, neutrophils, erythrocytes and platelets could not be analyzed. Analysis using t-tests demonstrated that the number of lymphocytes following PRFA was significantly lower compared with that prior to PRFA (P=0.007; Fig. 4A), and the CTC level in the decreased lymphocyte group was significantly higher compared with those in the increased lymphocyte group (Fig. 4B). This result revealed that a

Table VI. Blood indexes of partial patients and CTC changes mean value.

Characteristic	No. of patients (%)	Mean difference in CTCs[a]	P-value[b]
Tumor markers			
Increase	12 (30)	3.58	0.968
Decrease	11 (26)	3.45	
Monocytes			
Increase	30 (70)	3.6	0.766
Decrease	13 (30)	4.54	
Lymphocytes			
Increase	16 (37)	0.63	0.03
Decrease	27 (63)	5.81	
Lymphocytes percentage			
Increase	17 (40)	3.06	0.645
Decrease	26 (60)	4.42	
Total number of T cells			
Increase	15 (35)	2.8	0.165
Decrease	5 (12)	8.6	
CD3+CD4+ T cells			
Increase	15 (35)	3.6	0.709
Decrease	5 (12)	5.2	
CD3+CD8+T cells			
Increase	12 (28)	0.83	0.073
Decrease	8 (19)	8.75	
NK cells			
Increase	12 (28)	1.42	0.158
Decrease	8 (19)	7.88	

[a]Total CTC number following PRFA minus CTC number prior to PRFA. [b]Difference in total CTC number (post PRFA minus pre) divided into different groups according to each characteristic listed. P-values were obtained using an independent sample t-test or one-way analysis of variance test with post hoc test incuding LSD and Dunnett T3. PRFA, percutaneous radiofrequency ablation; CTCs, circulating tumor cells; CD, cluster of differentiation; NK, natural killer.

decrease in lymphocytes may be beneficial for the survival of tumor cells in the peripheral circulatory system. The immune system is known to inhibit the metastasis of cancer cells, and PRFA may affect the immune system, thus decreasing the number of lymphocytes, in turn decreasing the inhibitory effect on cancer cells. In terms of liver function indexes, it was demonstrated that alanine transaminase (ALT) and aspartate transaminase (AST) were significantly elevated ($P<0.001$) in patients following RFA (Fig. 4C and D), but their elevation was not significantly associated with changes in the CTC level (Table VII; Fig. 4E and F), and may instead be associated with the heat and mechanical damage of the liver cells during PRFA. Such damages to normal tissue may not lead to high CTC levels. No significant correlations were identified between immune cell subsets, tumor markers and changes in CTC levels.

Effect of treatment history on the change of CTCs following PRFA. In addition to PRFA factors, the patient's treatment history was analyzed, which included chemotherapy history and targeted therapy history (Table VIII). The following factors were taken into consideration: Chemotherapy or targeted therapy taken in the past month, the line of chemotherapy chosen, and the chemotherapy evaluation (partial response, stable disease, or progressive disease). In addition, whether chemotherapy regimens in patients with colorectal cancer affected CTC levels following PRFA were investigated. It was revealed that none of the aforementioned chemotherapy factors were significantly correlated with changes in CTC levels. As for targeted therapy, the use of targeted therapy within the past month did not affect the changes in CTC levels.

Discussion

The levels of CTCs have been reported to increase following RFA (33); however, no further studies have been performed to identify the factors that cause such increases. To the best of our knowledge, the present study is the first to investigate the association between liver tumor PRFA and changes in CTC levels. In addition, the current study is the first to investigate the variation in CTC phenotypes following liver tumor PRFA. Furthermore,

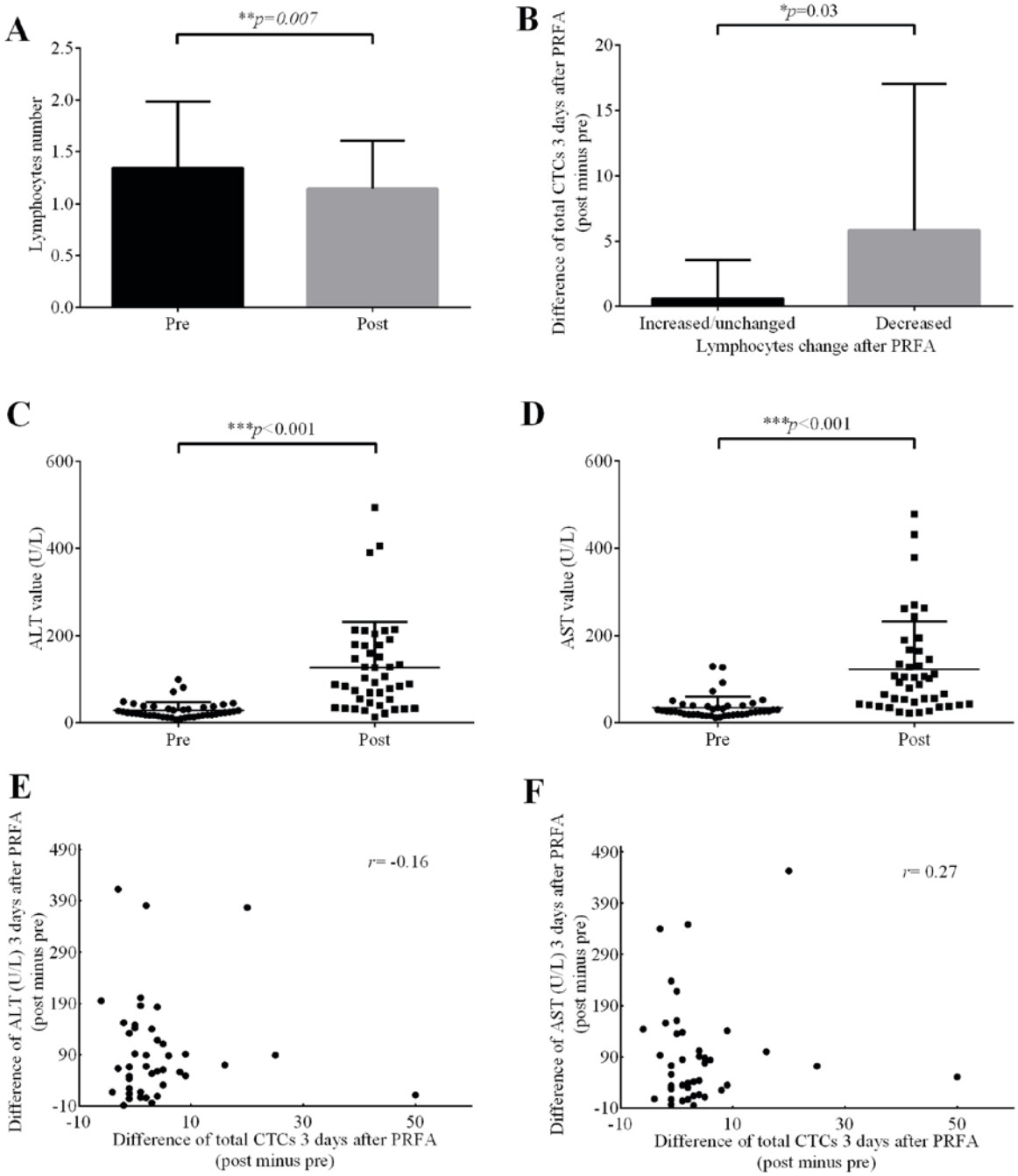

Figure 4. Effect of PRFA on lymphocyte numbers and ALT/AST value. (A) Significant difference in lymphocyte numbers between the pre-PRFA and post-PRFA group (P=0.007, paired sample t-test). (B) After dividing the difference in total CTCs (post PRFA minus pre) into two groups (increased and decreased lymphocyte numbers), a significant increase was identified in decreased lymphocyte group (P=0.03, independent sample t-test). (C) ALT and (D) AST levels increased significantly following PRFA (both P<0.001, paired sample t-test). Bivariate correlation analysis of changes in (E) ALT and (F) AST (post minus pre) vs. total CTC change (post minus pre), no correlation was identified (Spearman correlation coefficient, r=-0.16 and r=0.27, respectively). PRFA, percutaneous radiofrequency ablation; CTCs, circulating tumor cells; ALT, alanine transaminase; AST, aspartate transaminase.

it was confirmed that liver PRFA reduced the total number of lymphocytes, which may reduce the immune surveillance and killing function of tumor cells. This may be among the causes of elevated levels of total CTC in peripheral circulation. Further experiments are required to explore the association between changes in CTC levels and the immune system.

The CTC classification method used in the present study was based on RNA-ISH, which provides sufficient information at the transcriptional level. However, there may be certain limitations that exist at the protein level. Generally, compared with the post-transcriptional results of western blot analysis, RNA-ISH is more reliable.

A comprehensive analysis of various factors demonstrated that the PRFA factors did not correlate with the changes in CTC levels. Incomplete liver tumor ablation increases the risk for local recurrence and distant recurrence (34). To develop an efficacious PRFA plan, the ablation points, puncture times and treatment time should be decided based on the situation of individual patients. Certain doctors may doubt that increased puncture time or high number of ablation sites would further

Table VII. Liver function indexes ALT and AST change.

Characteristic	No. of patients (%)	Mean difference in ALT/AST[a]	P-value[b]	r-value[c]
ALT		98.1	<0.001	-0.16
Increase	43 (100)			
Decrease	0 (0)			
AST		88.8	<0.001	0.27
Increase	43 (100)			
Decrease	0 (0)			

[a]ALT/AST value post PRFA minus pre. [b]ALT/AST value post PRFA group and pre PRFA group. P-values were obtained using a paired sample t-test. [c]Spearman correlation coefficient by bivariate correlation analysis between the difference of total CTC number (post minus pre PRFA) vs. ALT/AST value (post minus pre PRFA). PRFA, percutaneous radiofrequency ablation; CTCs, circulating tumor cells; ALT, alanine transaminase; AST, aspartate transaminase.

Table VIII. Effect of drug treatment on the changes in CTCs following PRFA.

Characteristic	No. of patients (%)	Mean difference in CTCs[a]	P-value[b]
Chemotherapy history			0.522
No chemotherapy taken in the past month	10 (23)	4.32	
Chemotherapy taken in the past month	33 (77)	2.2	
Treatment			
5-FU + oxaliplatin	16 (37)	3.63	0.596
5-FU + irinotecan	10 (23)	6.0	
Chemotherapy line			0.388
Maintenance chemotherapy	2 (5)	0.5	
First-line chemotherapy	11 (26)	5.56	
Second-line chemotherapy	16 (37)	0.82	
Third-line chemotherapy	4 (10)	13.25	
Last therapeutic evaluation			0.185
PR	4 (10)	10.5	
SD	9 (21)	-0.67	
PD	15 (35)	5.6	
Targeted therapy			0.845
Not used in the past month	9 (21)	3.33	
Used in the past month	34 (79)	4.03	

[a]Total CTC number following PRFA minus CTC number prior to PRFA. [b]Difference in total CTC number (post PRFA minus pre) divided into different groups according to each characteristic listed. P-values were obtained using an independent sample t-test or one-way analysis of variance test with post hoc test incuding LSD and Dunnett T3. PRFA, percutaneous radiofrequency ablation; CTCs, circulating tumor cells; PR, partial response; SD, stable disease; PD, progressive disease.

increase the CTC level. Particular cases of needle tract seeding following percutaneous biopsy have been reported (35). In theory, physical factors, including heat injury and mechanical damage may also cause metastasis. RFA is a thermal ablation technique during which heat injury and mechanical damage to tumor tissue may lead to the shedding of CTCs from solid tumor and enter the circulatory system. A larger tumor burden comes with greater heat injury and mechanical damage, resulting in a higher possibility of CTC shedding into the circulatory system. Therefore, target tumor burden, numbers of ablation points, puncture times, treatment time and ablation location were all included in the present study. The evidence presented in the current study may aid in individually adjusting the PRFA factors to maximize the tumor ablation effect and decrease the risk of CTC level increase. The total number of CTCs increase following PRFA, but appropriate puncture times or tumor ablation points reduce any further increases to CTC levels.

In addition, no correlation was identified between elevated liver function indices, including ALT and AST, and elevated

CTC levels. The liver function index may be used to determine the degree of damage to normal liver cells as liver PRFA inevitably damages normal liver tissue. However, damage to normal liver tissue adjacent to tumor tissue do not result in changes to CTC levels. This may aid clinicians in creating a more comprehensive plan for PRFA of tumors with irregular shapes.

One previous study reported that the total number of CTCs increase following RFA (11). The current study further analyzed the changes in CTC phenotypes and confirmed that the increase in the total number of CTCs was primarily based on the mesenchymal phenotype of CTCs. Among the 43 patients included in the present study, 26 (60.5%) exhibited an increase in CTCs following PRFA. Among these 26 patients, 17 (65.4%) had more mesenchymal phenotype CTCs. PRFA leads to an elevated number of total CTC, and also to elevated mesenchymal phenotype CTCs. Epithelial CTCs transform into mesenchymal CTCs through EMT, and mesenchymal phenotype CTCs have an increased invasion capability (36). The mechanism by which mesenchymal phenotype CTCs increase following PRFA is unclear, but the EMT of CTC has been confirmed in the circulatory system (37), which may be one possible explanation. In addition, the second time point (day 3) by which blood samples were collected from the peripheral circulation was also a limitation. The EMT process of CTCs takes time, that is to say, different detection time points should indicate different CTC phenotypes. CTCs need to overcome several obstacles to colonize distant organs (38) and this process takes time. Thus, we hypothesized that a considerable proportion of epithelial phenotype CTCs on day 0 may transform into mesenchymal phenotype CTCs by day 3 via EMT. Thus, the total number of mesenchymal phenotype CTCs significantly increased on day 3. To depict the timeline of EMT of CTCs, phenotypes may be detected at different time points in follow-up studies.

Immunization has been demonstrated to inhibit certain malignant tumors (39). $CD8^+$ T-cells and natural killer (NK) cells have been proven to have significant effect on anti-metastatic immune surveillance (40). The current study focused on liver tumors and NK cells are abundant in the liver. One previous study has indicated that NK cell depletion in mice increase the occurrence of hepatic metastasis (41). In the analysis of the blood indexes in the present study, lymphocyte numbers were significantly lower 3 days after PRFA. Furthermore, the CTC level was significantly elevated in patients with reduced lymphocyte numbers. However, the P-values for $CD3^+8^+$ T cells and NK cells in certain patients were 0.073 and 0.158, respectively, and no statistical significance was identified. This may be associated with the small number of patients (14/43) tested for immune cell subsets.

Collectively, these results indicate that the increased number of CTCs following PRFA may be due to the decreased lymphocyte numbers, and more experiments are required to confirm this inference. Fewer lymphocytes mean a decrease in immune surveillance and killing function, which may lead to survival of more tumor cells in the circulatory system. In addition, EMT may result in a significant increase in the number of mesenchymal cells, which enhances the potential metastatic capability of cancers. Larger sample size studies as well as further studies investigating the mechanisms underlying this phenomenon are required. The results of the present study may aid clinicians in understanding the implications of elevated CTCs during liver tumor PRFA.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

WL, MS and YL designed and conceived this study. NH, CW and LL helped to collect patients' clinical data. HM and MZ helped to perform the statistical analysis, put together the figures and edit the manuscript. ZH and LS made substantial contributions to acquisition of data and drafting the manuscript.

Ethics approval and consent to participate

The present study was approved by the medical ethics committee of Nanfang Hospital, Southern Medical University (Guangzhou, China), and written informed consent was obtained from all patients.

Consent for publication

Consent for publication was received from patients.

Competing interests

All authors declare that they have no competing interests.

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