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Laparoscopic surgery minimizes the release of circulating tumor cells compared to open surgery for hepatocellular carcinoma

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Abstract
Background The aim of this study was to determine whether tumor manipulation enhances cancer cell release from the primary tumor in HCC patients and which surgical approach, open surgery or laparoscopic resection, is superior with respect to preventing tumor cells from scattering in the blood.
Methods A total of 26 HCC patients were prospectively randomized to receive either open surgery ($n = 14$) or laparoscopic surgery ($n = 12$). Blood samples were obtained at three time points: preoperative, postoperative, and 24 h after surgery. The $CD45^{-}/CD44^{+}/CD90^{+}$ cells were obtained and counted using quantitative flow cytometry. The serum levels of interleukin-6 (IL-6), interleukin-8 (IL-8), and tumor necrosis factor (TNF-α) were also compared between the two groups.
Results There was no significant difference between the laparoscopic and open groups in terms of patient characteristics. The levels of CCSCs increased immediately after surgical manipulation, and the laparoscopy group released fewer tumor cells into the blood stream. The amount of CCSCs in both groups decreased to reach a similar level 24 h after surgery. Both IL-6 and IL-8 increased after surgery, and the mean postoperative increases in IL-6 and IL-8 serum levels were significantly less in the laparoscopic group than in the open group. The TNF-α levels showed no differences at any time point.
Conclusions Our results showed that patients with laparoscopic surgery have lower IL-6, IL-8 secretion and less CTCs, which may suggest an advantage by restricting CTCs release and a preserved immune response. Further studies are needed to investigate the relationship between the number of CCSCs after surgery and long-term survival rates.

Keywords Laparoscopy · Hepatocellular carcinoma · Circulating cancer stem cells

Hepatocellular carcinoma (HCC) is one of the most common malignant tumors worldwide, and curative surgery is considered to be the main treatment for this disease. However, HCC is associated with a poor prognosis that can mostly be explained by early recurrence; approximately 40 % of patients develop recurrences within the first year after hepatectomy [1, 2]. It has been proven that tumor recurrence after resection is closely associated with circulating tumor cells (CTCs) [3]. CTCs are defined as tumor cells that have been shed from the primary tumor sites or metastases; they may attach to distant organs and have long been considered to be a marker of tumor aggressiveness [4, 5]. However, not all CTCs are able to trigger metastasis; only those CTCs that have cancer stem cell (CSC) characteristics can form ectopic metastases [3, 5, 6]. A prospective study reported that the CSC counts in the

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circulatory system exhibited a good correlation with liver tumor recurrence [3]. Therefore, it is reasonable to assume that early HCC metastases may be detected by isolating CSCs in the blood, and monitoring circulating cancer stem cell (CCSCs) changes is useful for efficacy assessments in HCC patients. There are numerous methods for detecting CSCs in the blood. Yang et al. found that $CD45^{-}/CD90^{+}/CD44^{+}$ cells could be detected in the peripheral blood from 90 % of HCC patients but not from the control group or from patients with liver cirrhosis [7, 8]. Their results have been verified in other studies [3].

Previous animal and clinical studies have demonstrated that CTCs can be shed into the circulatory system during tumor resection, and soft manipulation is able to reduce these micrometastases [9–12]. Laparoscopic liver surgery has evolved greatly over the past few decades, and it has been widely accepted as a classic minimally invasive surgical technique for various types of liver resection [13–16]. Studies have compared laparoscopic approaches to traditional open surgery in terms of operative blood loss, operation time, postoperative pain, and length of hospital stay [17–24]. No study has ever compared these two procedures regarding the prevention of CTC release in HCC patients.

In our study, we investigated whether liver surgery promotes the release of CSCs in primary HCC patients and which surgical approach, laparoscopy or open surgery, can reduce the release of CSCs into the blood stream and preserve immune function better.

Patients and methods

Ethics

This study was approved by the medical ethical committees of our institution. The advantages and risks of the study were explained to the patients before operations, and all blood samples were obtained with patient consent.

Patients

Between October 2012 and June 2013, 26 patients who underwent laparoscopic hepatectomy ($n = 12$) or open liver resection ($n = 14$) for HCC in the Department of Hepatobiliary Surgery at The Sun Yat-sen Memorial Hospital of Sun Yat-sen University were prospectively enrolled in the study (Table 1). The inclusion criteria included compensated cirrhosis or noncirrhotic liver, a platelet count of 100×10^9/l or higher, and a tumor location that was not in contact with the portal pedicle or hepatic veins. The exclusion criteria were distant metastases, an American Society of Anesthesiologists (ASA) score >3 points, and age >80 years. Patients who underwent palliative resection only and those who received intraoperative blood transfusions were also excluded.

All procedures were performed by the same surgical team, and the same surgical and oncological principles were followed in both groups. Furthermore, the patients in the laparoscopic surgery group (lap) and those in the open surgery group (open) received similar preoperative and postoperative management. Patient characteristics, age, Child status, tumor size, blood loss, and operation time were separately collected and analyzed.

Outcome measures

Collection of blood samples

On the day of the operation, 12 ml of peripheral blood was obtained from all patients at three time points: immediately before skin incision (preoperative), immediately after closure of the skin incision (postoperative), and 24 h after surgery. Blood samples (10 ml) were collected in BD Vacutainer tubes (Becton–Dickinson, Franklin Lakes, NJ) containing EDTA for CTC detection, and additional blood samples (2 ml) were used to assay the IL-6, IL-8, and TNF-α serum levels.

Inflammatory response

All serum IL-6, IL-8, TNF-α samples were stored at −80 °C until analysis. Samples were measured using commercially available enzyme-linked immunosorbent assay kits (Koma Biotech, Korea).

CCSC selection and detection

Mononuclear cells were separated from the EDTA blood using Ficoll-Paque PLUS (GE Healthcare Bioscience AB, Uppsala, Sweden) density gradient centrifugation before flow cytometry analysis was performed. In a 10-ml test-tube, 2 ml of anticoagulant-treated blood and an equal volume of balanced salt solution were combined (final volume: 4 ml). The blood and the buffer were mixed by drawing the components in and out of a Pasteur pipette. Ficoll-Paque PLUS (4 ml) was added to the centrifuge tube. The diluted blood sample (4 ml) was carefully layered onto the Ficoll-Paque PLUS. When layering the sample, the Ficoll-Paque PLUS and the diluted blood sample were not mixed. Each tube was centrifuged at $400 \times g$ for 30–40 min at 18–20 °C. The upper layer was removed, leaving the lymphocyte layer undisturbed at the interface. The lymphocytes were then washed twice with 6 ml of a balanced salt solution. The supernatant was discarded, and the lymphocytes were resuspended in 1 ml of Stain Buffer (Becton–Dickinson). A precise volume of 100 µl cells was then added to a Tru-COUNT Tube (Becton–Dickinson).

Table 1 Clinical characteristics of 26 patients with primary liver cancer

	Laparoscopic ($n = 12$)	Open ($n = 14$)	P value
Age (year), median (range)	49 (27–72)	57 (33–73)	0.519
Child status			
A	10	12	
B	2	2	0.867
Tumor size (cm)	5.96 ± 2.82	6.23 ± 3.16	0.821
Operation time (min)	201 ± 64	160 ± 54	0.084
Blood loss (ml)	373.3 ± 310.3	410.7 ± 331.7	0.771
TNM stage			
I	2	2	
II	10	12	
III	0	0	
IV	0	0	0.867
Type of liver resection			
Major hepatectomy	5	6	
Bisegmentectomy	3	3	
Segmentectomy	1	2	
Wedge resection	3	3	0.965

Continuous variables are expressed as mean ± SD

The isolated cells were labeled with CD45-PE, CD44-APC, and CD90-FITC (Miltenyi Biotec, GmbH, Germany). We used appropriate isotypes of nonrelated antibodies as controls. Each tube was then capped, vortexed gently, and incubated for 30 min in the dark at 4 °C. Then, 340 µl of 1 × Stain Buffer was added to the tubes, and they were vortexed gently. The samples were analyzed using an Accuri C6 Flow Cytometer (Becton–Dickinson Immunocytometry Systems, San Jose, CA). The BD software program (BD FACSComp™, version 2.0) was used to automatically calculate the absolute cell counts in 2 ml of peripheral blood. Based on the number of CCSCs in the 2-ml blood samples, we estimated the number in 10-ml blood samples.

Statistical analysis

All statistical analyses were conducted using SPSS version 13.0 for Windows (SPSS, Chicago, IL, USA). Continuous variables were compared using Student's t test and the Mann–Whitney U test, and the Chi-squared and Kruskal–Wallis H tests were applied for categorical variables when appropriate. For all analyses, P values less than 0.05 were considered statistically significant.

Results

Patient characteristics

A total of 26 patients were enrolled in this prospective clinical study. The median age of the patients was 55.5 years (range 27–73 years), and all of the patients were male. There were no open conversions in the laparoscopy group and no intraoperative complications (including the need for a blood transfusion) in any of the patients in either group. The patient characteristics are listed in Table 1. There were no significant differences between the laparoscopic and open groups in terms of patient age, child status, tumor size, blood loss, and operation time. Furthermore, the TNM stage and type of liver resection were comparable.

Inflammatory response

The IL-6, IL-8, and TNF-α levels were similar between groups before the operation (Table 2). The mean increases in the postoperative serum levels of IL-6 and IL-8 in the laparoscopic group were significantly less than those in the open group (73.60 vs. 116.19 pg/mL, respectively, for IL-6; 36.55 vs. 47.37 pg/mL, respectively, for IL-8). Only the IL-6 levels in the laparoscopic group showed a significantly diminished increase 24 h after surgery compared to that of the open group (121.76 vs. 192.37 pg/mL, respectively). The TNF-α levels did not differ between the two groups at any of the time points examined (Table 2; Fig. 1).

The effects of the two approaches on circulating stem cell levels

Before surgery, the presence of CCSCs could only be detected in the blood samples from 3 patients (25 %) in the laparoscopy group, and the median count in all of those blood samples was 0 cells (range 0–50 cells). Of the 14 patients who underwent open surgery, 3 (21 %) had blood samples that were positive for CCSCs, and the median count in this

Table 2 Comparison of immunologic markers and CCSCs between laparoscopy (lap) and open surgery (open)

Variable	Group	preoperative	Postoperative	24 h after surgery
IL-6 (pg/mL)	Lap	66.20 ± 25.09	73.60 ± 30.13*	121.76 ± 37.00*
	Open	63.68 ± 35.05	116.19 ± 26.59*	192.37 ± 59.96*
IL-8 (pg/mL)	Lap	23.89 ± 13.02	36.55 ± 15.74*	55.04 ± 30.51
	Open	20.49 ± 12.91	47.37 ± 15.29*	54.52 ± 24.88
TNF-α (pg/mL)	Lap	40.52 ± 27.85	49.17 ± 29.26	66.86 ± 34.89
	Open	31.46 ± 12.53	64.20 ± 33.01	67.12 ± 32.30
CCSCs	Lap	0 (0–50)	88 (0–190)*	5 (0–77)
	Open	0 (0–38)	164 (0–495)*	4 (0–148)

The average number of CCSCs per 10 ml blood counts is descriptive as median and range in the table

Asterisk (*) indicates $p < 0.05$ laparoscopic versus open groups

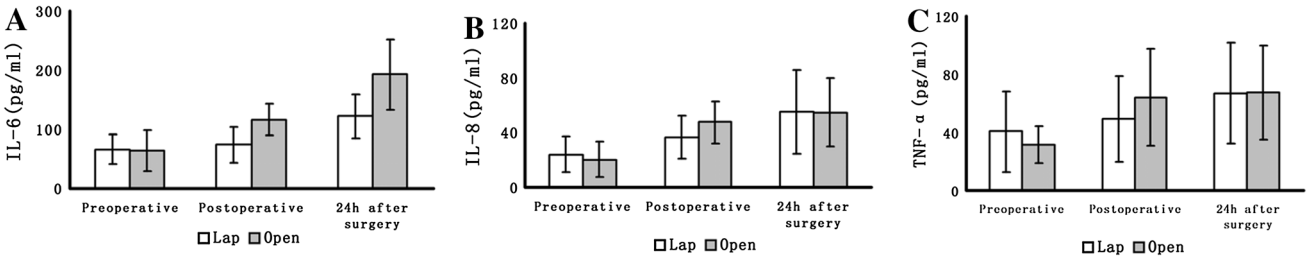

Fig. 1 The changes in immunologic markers (**A**: IL-6; **B**: IL-8; **C**: TNF-α) during the period of observation in both laparoscopy group (lap) and open surgery group (open)

group was 0 cells (range 0–38 cells). No significant difference was found in the level of CCSCs between the two groups before surgery manipulation ($P = 0.705$; Table 2; Fig. 2).

Table 2 and Fig. 2 indicated a clear trend toward an increased level of CCSCs in the two groups after tumor resection, and a comparison of the median levels revealed a significantly lower increase ($P = 0.041$) following laparoscopic surgery (88 cells; range 0–190 cells) compared to open surgery (164 cells; range 0–495 cells). The flow cytometry graphs (Fig. 3) indicate that the levels of CCSCs varied in patients before and after surgery.

The CCSC counts decreased substantially 24 h after surgery in most patients from both groups (Table 2; Fig. 2), and a comparison between the laparoscopic and the open surgery groups at this time point showed no difference in the level of CCSCs ($P = 0.806$, Table 2). Importantly, both before and after surgery, no CCSCs were found in 1 patient (8 %) from the laparoscopic surgery group and 2 patients (14 %) from the open surgery group.

Discussion

Surgical liver resection is the most effective therapy for early stage HCC patients [25]. Classical open surgery offers the following advantages: good tactile sensation, facilitation of liver mobilization, bleeding control, and

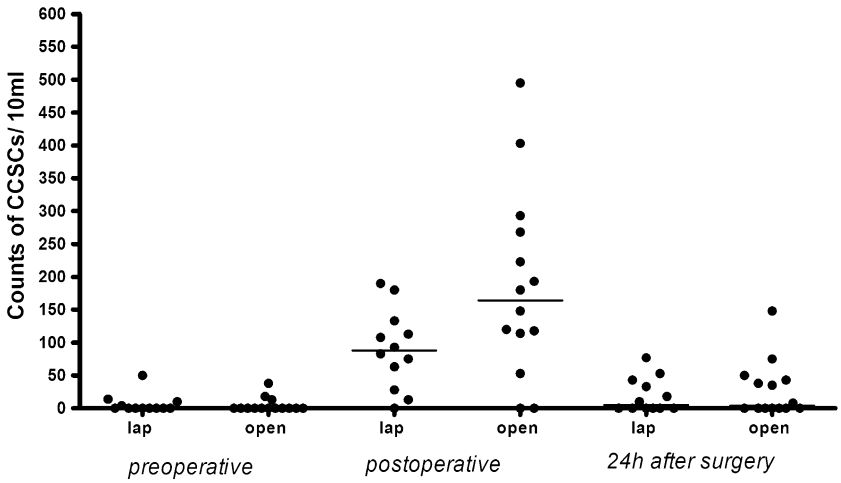

Fig. 2 The counts of CCSCs for each sample in laparoscopy group (lap) and open surgery (open) group at three time points. Spot (*filled circle*) stands for the estimated numbers of CCSCs in 10-ml blood samples. Transverse line (*horizontal line*) stand for the median numbers of CCSCs for all samples in this group

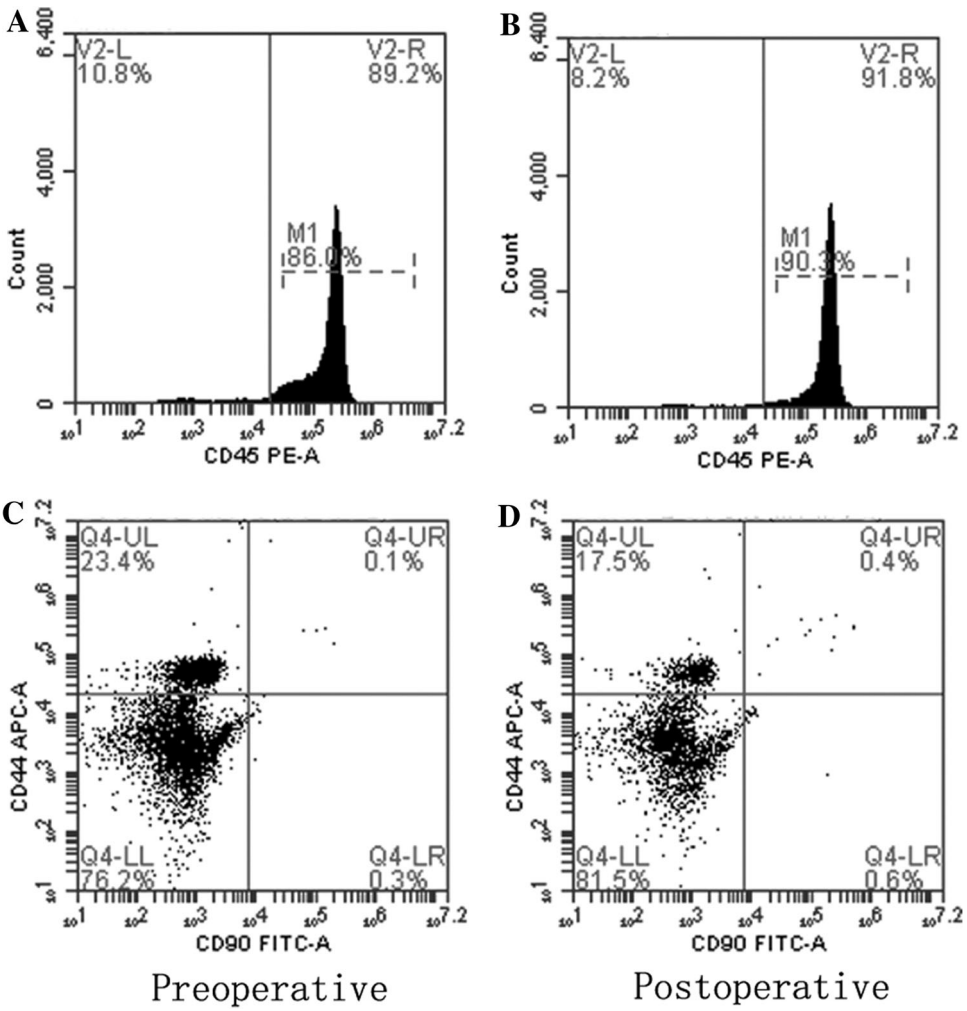

Fig. 3 Identification of circulating cancer stem cells in hepatocellular carcinoma patients by flow cytometry. **A/B**: The percentage of $CD45^-$ cells at preoperative time point (**A**) and at postoperative time point (**B**); **C/D**: The percentage of $CD44^+$ $CD90^+$ cells before surgery (**C**) and immediately after surgery (**D**)

possible improvements in diagnosis and staging accuracy for malignancies [26]. Laparoscopic surgery, by decreasing trauma, offers the following advantages: decreased postoperative pain, shorter hospital stay, and a faster return to normal activity [24]. Despite these advantages, patients who undergo curative liver resection still face a high risk of tumor recurrence due to the presence of CTCs, which have long been considered to be a marker of tumor aggressiveness [2, 5]. But not all CTCs are able to form ectopic metastasis; only a subset of CTCs exhibit CSC characteristics and are known as CCSCs [5].

In previous studies, researchers have focused on identifying the specific tumor markers or molecular markers that may correlate with post-hepatectomy recurrence. α-Fetoprotein (AFP) is believed to be a classic prognostic predictor of HCC [27]. However, this marker lacks sensitivity and specificity [28, 29]. For CTCs, the extremely small number of CTCs in blood samples makes them a difficult marker to study [30, 31]. To date, several methods have been employed to quantify the number of CTCs in the blood. Among them, there're methods based on RT-PCR-based assays which are unable to accurately count the number of CTCs. Another method is called the Cell Search system, which is based on EpCAM anti-body-coated magnetic beads, has been approved by the U.S. Food and Drug Administration for the detection of CTCs in breast cancer, colon cancer, and prostate cancer. The use of the CellSearch system is not appropriate for detecting CTCs in HCC patients because only approximately 35 % of HCC cases express EpCAM [32, 33]. Therefore, a sensitive and specific method is needed to detect CTCs specifically in HCC patients. Fan et al. recently found that the number of circulating cancer stem cells ($CD45^-/CD90^+/CD44^+$) in the blood stream showed a good correlation with the post-hepatectomy recurrence of HCC; CCSCs >0.01 %, tumor stage, and tumor size were independent factors that predicted the recurrence-free survival. The prediction ability of this factor seems to be higher than that of conventional methods. In this study, we used a similar method to count the CCSCs and aimed

to determine the relationship between CCSCs and the applied surgical technique.

Our results indicated that the basal level of CCSCs was similar in both groups before surgery. This level can increase immediately after surgical manipulation. It is clear that the level of CCSCs was affected by the nature of the surgical intervention being applied. Based on a tumor-bearing mouse model, Juratli et al. recently discovered that tumor manipulation, including tumor palpation, pressure, biopsy, and laser treatment, can increase the release of cancer cells from the primary tumor into the circulatory system [34]. Human studies suggest that malignant tumor cells are released after surgical manipulation involving colorectal cancer resection. Akiyoshi et al. further demonstrated that laparoscopic surgery, a minimized surgical manipulation, reduced the CTC release from the primary tumor comparing to that of open surgery [12, 35, 36]. Consistent with these results, we found that patients in the laparoscopy group released fewer tumor cells into peripheral blood during liver resection. We assumed that open surgery may increase the release of malignant cells during surgical procedures. Laparoscopic surgery, however, facilitates a more gentle surgery and less surgical manipulation of the tumors, which may be thought of as a "no-touch" isolation procedure and may decrease the risk of tumor recurrence.

In this study, we also observed changes in IL-6, IL-8, and TNF-α levels after surgery. The increased serum levels of IL-6, IL-8, and TNF-α in patients were correlated with early tumor cell dissemination and reduced survival [37–42]. In addition, these changes are thought to influence tumor recurrence by changing the tumor microenvironment [41, 42]. We found that the postoperative increases in IL-6 and IL-8 levels in the laparoscopic group were significantly diminished relative to those in the open surgery group. IL-6, which stimulates angiogenic pathways and enhances vascular endothelial growth factor, plays a crucial role in tumor growth, recurrence, and metastasis [43]. In patients undergoing laparoscopic surgery for cancer, the preservation of angiogenic mechanisms may strengthen oncological control. This potential advantage has been observed in colon cancer surgery and nephrectomy [43, 44]. It is believed that improvements to the preservation of immune function in laparoscopic surgery could prevent tumor nestling and formation of distant metastases [35, 45]. The levels of serum TNF-α increased in both the open and laparoscopic groups after surgery. However, no significant difference was found between the two groups. TNF-α is produced mostly by T-helper 1 (Th-1) cells, and our results suggest that Th-1 cells might be activated to a similar degree in both groups. Differences in the immune system between two groups after surgery may not fully explain the number of CCSCs in both groups decreased to the same level 24 h after surgery. The number of CCSCs 24 h following surgery reflects the combined results of two processes: release and depletion. CTCs depletion might be caused not only by immune system targeted destruction but also by apoptosis and metastasis to other organs. Nature killer (NK) cells are cytotoxic lymphocytes that contribute to destruction of cancer cell. Low NK cells activity during the postoperative period—associates with higher cancer recurrence rate [45]. Moreover, Smerage et al. suggested that—CTCs undergo apoptosis either in the tumor or as a function of separation from the parent cancer. Moreover, the relative percent of apoptosis was inversely associated with CTCs levels [46]. However, the role that apoptosis and immune response play in the CTCs depletion process, especially in hepatocellular carcinoma, is still poorly understood. Further analysis about CTCs depletion and apoptosis are required.

There are some limitations. Firstly, despite the high sensitivity of this method in detecting CCSCs, the overall detection accuracy did not reach 100 %. Secondly, although we selected patients according to strict principles and all operations were performed by the same surgical team, we cannot completely exclude the impact of variations in clinical situations, such as individual patient differences.

In conclusion, our results showed that patients with laparoscopic surgery have lower IL-6, IL-8 secretion and less CTCs, which may suggest an advantage by restricting CTCs release and a preserved immune response. Further studies are needed to investigate the relationship between the number of CCSCs after surgery and long-term survival rates.

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