

# Circulating Tumor Stem Cells and Circulating Tumor Cells are Prognostic Biomarkers for Patients with Metastatic Colorectal Cancer

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## Abstract

**Introduction:** Circulating tumor cells (CTCs) in blood have been studied as predictors of recurrence and the prognosis of various solid cancers. Circulating tumor stem cells (CTSCs) are involved in the metastatic recurrence of cancer however, no clinical finding regarding CTSCs is present in the literature. The aim of the study was to determine the potential of CTC and CTSC measurements as biomarkers for colorectal cancer.

**Methods:** This is prospective single-center observational study. We included 120 patients with colorectal cancer between November 2008 and 2014 in this study. CTCs and CTSCs were isolated via immunomagnetic separation and stained with fluorescently labeled monoclonal antibodies; their levels were then measured.

**Results:** CTCs were detected in 45 of 120 patients. In patients with Stage IV cancer, 14.8% (12/81) were positive and they had a significantly worse outcome ( $P = 0.00021$ ). In addition, CTCs were measured in specimens obtained during apheresis from six patients with Stage IV cancer even though all six patients were positive for CTCs. Furthermore, the prognosis of patients with mCRC who tested positive for CD133 or CD44 as well as for CTSCs was significantly poorer than for the 14 subjects with mCRC who did not test positive for CD133 or CD44 ( $p = 0.0183$ ).

**Conclusion:** The potential of CTCs as a biomarker was negligible in patients with Stage I–III cancer. CTCs appeared to be potential biomarkers for poor prognosis in patients with Stage IV cancer. Therefore, the presence of CTSC in metastatic colorectal cancer cases can be considered as a biomarker for poor prognosis.

**Keywords:** Circulating tumor cell; Circulating tumor stem cells; CellSearch system; Colorectal cancer

## Introduction

In recent years, clinical applications in a variety of solid cancers have incorporated Circulating Tumor Cell (CTC) count as an index for predicting prognosis and recurrence and for evaluating the response to chemotherapy.

Among CTC-counting modalities, the CellSearch® system (Janssen, Raritan, NJ, US) has been approved by the US Food and Drug Administration for predicting and evaluating the probability of Progression-Free Survival (PFS) and Overall Survival (OS) during chemotherapy for patients with recurrent and progressive breast cancer [1] and prostate cancer [2]. Several clinical reports

have demonstrated that CTC count during chemotherapy for breast cancer acts as a useful criterion for selecting therapeutic modalities. Reports have indicated that CTC count has already been clinically applied as a biomarker for personalized therapy [3].

CTC count is also anticipated to be an exceptionally useful clinical biomarker for diagnosing recurrence in colorectal cancer. For instance, if CTC count can be applied to determine indications for adjuvant chemotherapy after surgical excision or to evaluate the effects of chemotherapy for progressive recurrent cancer, effective and efficient treatment regimens may be developed.

A previous study using the CellSearch system for metastatic Colorectal Cancer (mCRC) has reported a significant increase in the duration of PFS and OS following the initial administration of

chemotherapy drugs in patients with CTC count of  $\geq 3$  compared with that in patients with CTC count of  $\leq 2$ . These results indicate that CTC count can be used as an index for predicting the efficacy of chemotherapy [4]. However, its clinical significance beyond this use remains to be fully established.

Circulating tumor stem cells (CTSCs), CTC variants in which cancer stem cell markers are expressed in high quantities, are involved in the metastatic recurrence of cancer [5]. In metastatic breast cancer, the breast cancer stem cell marker ALDH1 is expressed in high quantities in CTCs [6]. In colorectal cancer, CD133 and CD44 are cancer stem cell markers [7-10]. However, no clinical finding regarding CTSCs is present in the literature.

In the present study, we determined CTC and CTSC counts in patients with colorectal cancer using the CellSearch system and evaluated these counts as predictive and prognostic indices for cancer progression and recurrence following radical surgical excision.

## Materials and Methods

### Study Design

This is prospective single-center observational study.

### Patients

In total, 120 patients with colorectal cancer [77 men and 43 women; average age, 67.0 (29–93) years] who underwent CTC count between November 2008 and October 2014 were enrolled. This study was approved by the Ethics Committee of Tokyo Medical University Hospital. Informed consent was obtained from all patients. Thirteen, 15, and 16 of the study subjects had stage I, II, and III cancer, respectively, according to the eighth edition of the International Union Against Cancer TNM classification staging system. Seventy-six of the total number of patients had metastatic colorectal cancer (mCRC), 19 of whom had received radical surgical therapy.

Preoperative cancer-bearing samples were collected from patients with stage I–III cancer, and those from patients with mCRC were collected prior to treatment.

### CTC-counting Method

The CellSearch system was employed for performing the CTC count; 7.5 ml of peripheral blood was collected from each subject and stored in a Cellsave tube (Janssen, Raritan, NJ, US). Each storage tube contained anticoagulant and cell preservation agents (composition: 4.6% EDTA, 36% cell preservation solution, 0.36% PEG, and 0.46% inactive ingredients). Each blood sample was mixed by immediately inverting after collection and was analyzed within 72 h. Next, the stored whole blood was mixed with a diluting solution (0.5% PBS and 0.1%  $\text{NaN}_3$ ) before centrifuging

at 800×g for 10 min at room temperature. During this process, the brakes on the centrifuge were released. After centrifugation, each sample was placed in a CelltracksAutoprep system along with a CellSearch epithelial cell detection kit (Janssen, Raritan, NJ, US), thereby initiating the completely automatic isolation and staining of CTCs. Subsequently, cells were photographed and observed using a Celltracks Analyzer II system (Janssen, Raritan, NJ, US). Based on macroscopic observation, cells counted as CTCs were those with intact cytokeratin, >50% DAPI staining in DAPI and CK images, no overlap between CK images and CD45 images, and CK images measuring  $\geq 4 \mu\text{m}$ .

### CTSC-counting Method

Samples of the 50-fold diluted anti-human/mouse CD133 (FITC conjugated; eBioscience) or CD44 (FITC conjugated; biorbyt) antibody were loaded onto the dedicated section of the epithelial cell detection kit cartridge from CellSearch for separation, and CD133- and CD44-positive cells were simultaneously stained. Subsequently, the cells were photographed and stained using the Celltracks Analyzer II (Janssen, Raritan, NJ, US). Of the cells identified as CTCs based on macroscopic observation, those that stained positively with CD133 and CD44 were counted as CTSCs.

### Statistical Analysis Method

Previous research on colorectal cancer has reported a CTC count cut-off of 3 cells/7.5 ml [4]. Therefore, subjects with a CTC count of  $\geq 3$  cells/7.5 ml in the present study were considered CTC-positive. Subjects who exhibited  $\geq 1$  CTSC/7.5 ml of sample were analyzed as CTSC-positive.

The duration of survival of each subject was recorded, starting from the date of blood sample collection. Statistical analysis of survival was performed using the Kaplan-Meier estimator and the log-rank test. Statistical analyses of CD133- and CD44-positive cell counts were performed using Fisher's exact probability test. A p-value of  $<0.05$  was regarded as statistically significant.

## Results

Of the 120 subjects, 45 (37.5%) exhibited one or more CTCs per sample, 12 (10.0%) of whom were diagnosed as CTC-positive, with the detection of three or more CTCs. Two samples were successively collected from each of the 16 subjects, four of whom (25%) displayed three or more CTCs in one sample (CTC-positive) but tested CTC-negative in the second sample.

In an analysis using clinical stage, 10 subjects with stage I–III cancer (22.7%) exhibited one or more CTCs but none experienced metastatic recurrence. Only one subject with stage I cancer (8.3%) tested positive by exhibiting three or more CTCs. Although one subject with stage I cancer and one with stage III cancer

experienced relapses, no CTC was detected prior to resection of the primary lesion.

Of the 76 subjects with mCRC, 35 (46.1%) exhibited one or more CTCs, 11 of whom (14.5%) were diagnosed as CTC-positive, with three or more CTCs (Table 1).

CTC-positive (≥3)

Stage	Positive rate (%)	Number of positive cases	Number of cases
I	7.69	1	13
II	0	0	15
III	0	0	16
IV	14.5	11	76

Table 1: CTC-positive (≥3) rate by stage.

Of the 76 subjects with mCRC, 11 (14.5%) were diagnosed as CTC-positive, with three or more CTCs. A CTC-positive diagnosis was extremely rare among subjects with stage I-III cancer.

Median survival of the 11 CTC-positive subjects was 13.0 (0.2-36.4) months. Survival analysis findings revealed a significantly poor prognosis in the CTC-positive group (p = 0.000152) (Figure 1). No correlation was observed between the CTC count and the duration of survival in the CTC-positive group. CTSC was counted for 22 cases (Table 2).

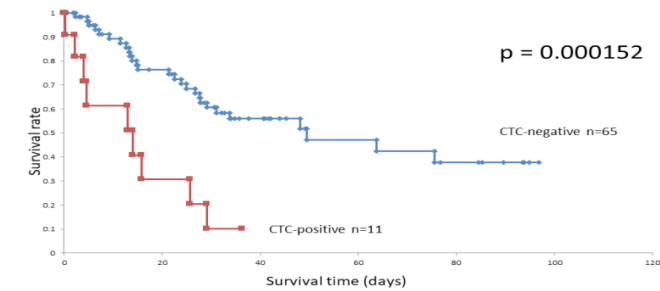


Figure 1: Overall survival based on CTC-positivity.

Median survival of the 11 CTC-positive subjects was 13.0 (0.2-36.4) months. Survival analysis findings revealed a significantly poor prognosis in the CTC-positive group (p = 0.000152).

CTSC-counting

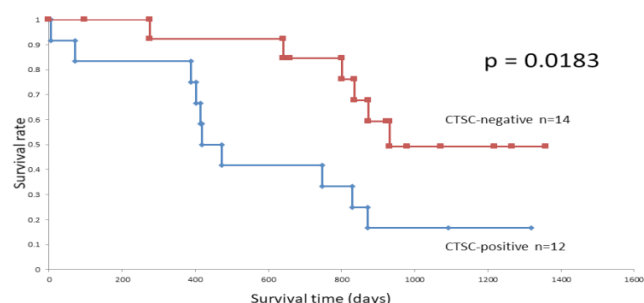
	CTC	CD44	CD133	CTC	CD44	CD133	CTC	CD44	CD133
1	0	0	-	1	-	1			
2	0	0	-	1	-	1			
3	0	0	-	1	-	0			
4	0	-	-	3	0	-	0	-	0
5	0	0	-	3	3	-			
6	1	1	-	0	0	-			
7	1	1	-						
8	1	0	-						
9	1	0	-						
10	2	2	-	0	-	0			
11	2	1	-	1	-	0			
12	2	2	-	2	-	1			
13	2	1	-	3	-	0			
14	2	2	-						
15	2	-	-						
16	3	-	-	1	1	-	1	-	1
17	3	2	-	7	-	1			
18	5	-	-	5	1	-			
19	10	6	-	12	-	0			
20	19	4	-						
21	50	-	-	20	1	-	30	-	2
22	273	-	-	394	1	-	428	-	0

Table 2: CTSC-counting. CTSC was counted for 22 cases.

Of the 11 CTC-positive subjects, six (54.5% positive rate) also exhibited CD133-positive cells. The CD133-positive rate was 66.7% (4/6) among subjects who exhibited less than three CTCs. This was not significantly different from the 40.0% (2/5) positive rate among the five subjects who exhibited three or more CTCs. However, 57.1% (4/7) of the subjects who exhibited less than three CTCs tested CD133-positive for all CTCs detected. This was significantly different from the 0.625% (3/480) CD133-positive rate among subjects with three or more CTCs (p = 0.0000048).

Meanwhile, 15 of the 18 subjects who exhibited one or more CTCs tested positive for CD44 (83%), with at least one CD44-positive cell detected. Among these 15 subjects, 10 exhibited less than three CTCs, and 80% were CD44-positive (8/10). This was not significantly different from the 87.5% (7/8) CD44-positive rate among the eight subjects with three or more CTCs. However, 73.3% (11/15) of the subjects with less than three CTCs tested CD44-positive for all detected CTCs, which was significantly different from the 3.94% (18/457) CD44-positive rate among subjects with three or more CTCs (p = 0.0000012).

Furthermore, the prognosis of subjects with mCRC who tested positive for CD133- or CD44 as well as for CTSCs was significantly poorer than the 14 subjects with mCRC who did not test positive for CD133 or CD44 (p = 0.0183) (Figure 2).



**Figure 2:** Overall survival based on CTSC-positivity.

The prognosis of subjects with mCRC who tested positive for CD133 or CD44 as well as for CTSCs was significantly poorer than the 14 subjects with mCRC who did not test positive for CD133 or CD44 ( $p = 0.0183$ ).

## Discussion

The CellSearch system used in this study is fairly easy to use and enables semiautomatic image analysis of 7.5 ml of peripheral blood collected using a simple method. With this system, researchers can detect CTCs from the epithelial cell adhesion molecule (EpCAM), which is a transmembrane glycoprotein involved in cell adhesion, cell growth, and tumor progression. EpCAM is expressed in most epithelial tumor cells, including colorectal cancer cells.

Studies on mCRC using the CellSearch system have reported a significant increase in the duration of PFS and OS in patients with a less than three CTCs compared with that in patients with three or more CTCs [4,11].

In this study, the prognosis of subjects exhibiting three or more CTCs was significantly poorer than that of subjects with less than three CTCs. Therefore, being CTC-positive may serve as a biomarker for the prognosis of patients with mCRC.

However, a CTC-positive diagnosis was extremely rare among subjects with stage I-III cancer, and its effectiveness as a biomarker for predicting recurrence was not supported by the CTC count performed prior to the radical surgical operation. Among subjects with stage I disease who had undergone curative resection, only one (an 86-year-old woman) tested CTC-positive, with 3 CTCs/7.5 ml of sample. This patient did not receive chemotherapy after the radical surgical excision and survived for 5 years without any relapse. Therefore, being CTC-positive was not significant in this case.

A previous study has reported that CTSCs, CTC variants in which cancer stem cell markers are expressed in high quantities, are involved in the metastatic recurrence of cancer [5]. In the study using CTC-chip, OS and DFS of patients who were positive for CD133 were significantly worse in patients who require adjuvant

chemotherapy for CRC [12]. In the present study, all detected CTCs were subjected to staining for CD133 and CD44, which are stem cell markers for colorectal cancer. Consequently, we discovered that many subjects with a lower CTC count tested positive for either CD133 or CD44 and exhibited a higher proportion of CTSCs, with the ratio substantially decreasing with increase in the CTC count.

Furthermore, the 12 CTSC-positive subjects showed a significantly poorer prognosis than the 14 CTSC-negative subjects. This finding suggests that the presence of CTSCs serves as a biomarker for poor prognosis even in CTC-negative cases (CTC count of  $\leq 3$ ).

Paget proposed the “seed and soil” hypothesis [13]. If CTCs correspond with the “seed” in this hypothesis, it is theoretically possible that they are present in every metastasizing carcinoma. However, with a half-life of only a few hours, CTCs do not exist in the bloodstream for a long period of time. In addition, CTCs seem to have detection limits in peripheral blood when it is refluxed into the portal blood system in patients with colorectal cancer. Matsusaka et al. have reported that approximately 19% of subjects with mCRC are CTC-positive, with a CTC count of  $\geq 3$  [11]. This percentage was not higher than that in subjects with other types of carcinoma. In this study, the detection rate of CTCs in peripheral blood samples was as low as 14.5%. This low detection rate may be a challenging factor for counting CTCs in patients with mCRC.

The CellSearch system only uses small-volume samples to count CTCs. Therefore, false-positive or false-negative results may occur in patients with a small absolute number of CTCs. To address this problem, we counted CTCs in two samples successively collected from 16 subjects. Among these, four subjects (25%) exhibited three or more CTCs in one sample but tested negative in the second sample, with less than three CTCs detected. This finding suggests that CTC count using the CellSearch system is prone to false positives or false negatives in colorectal cancer. Increasing the number of measurements or the volume of a single sample may aid in improving accuracy.

In recent years, it has been proposed that the epithelial-mesenchymal transition (EMT) plays a crucial role in cancer metastasis [14]. The EMT is a state in which epithelial cancer cells morphologically transform into mesenchymal cells during metastasis; through the EMT, cancer cells acquire the morphology and property of mesenchymal cells and become capable of metastasis. Through the process of metastasis, cancer cells that enter the EMT transform into EMT-associated or epithelial-like CTCs, thereby losing the characteristics of epithelial cells. At the same time, EpCAM expression suddenly decreases or disappears altogether. In principle, the CelltracksAutoprep system cannot detect cells that do not express EpCAM. Therefore, one future challenge will be to construct a system capable of detecting CTCs in the EMT.



In patients with colorectal cancer, the CellSearch system can currently count only a small number of CTCs, effectively limiting the usefulness of CTC count as a biomarker. Nevertheless, CTC count has the advantage of being a non-invasive method of molecular biological evaluation capable of monitoring tumor cells over time, which is a significant contribution to the increasingly important practice of personalized medicine in colorectal cancer. Furthermore, to the best of our knowledge, using the CellSearch system, the present study demonstrated, for the first time, that CTSCs can serve as a biomarker for poor prognosis in patients with mCRC. As technological advances begin to provide solutions to the current drawbacks of counting CTSCs and CTCs, they will play increasingly important roles in clinical treatments for colorectal cancer in the future.

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## Conflicts of Interest

The authors declare that they had no conflict of interest.

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