A new size-based platform for circulating tumor cell detection in colorectal cancer

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Abstract

Purpose: Circulating tumor cells (CTCs) may play a significant role in cancer progression and metastasis. However, the ability to detect CTCs is limited, especially in cells undergoing epithelial-mesenchymal transition. In this study, we evaluated a new size-based CTC detection platform and its clinical efficacy in colorectal cancer.

Materials and Methods: Blood samples were obtained from 76 patients with colorectal cancer and 20 healthy controls for CTC analysis. CTCs were enriched using a high-density microporous chip filter and were detected by a four color staining protocol including DAPI for nucleated cells, CD45 mAb as a leukocyte marker, and EpCAM mAb or CK mAb as an epithelial cell marker. CTC positivity was defined as DAPI+/CD45-/EpCAM+ or CK+ cells and clinical outcomes of patients were analyzed according to CTC counts.

Results: CTCs were detected in 50 patients (65.8%) using this size-based filtration platform. CTC-positive patients were more frequently identified with a high level of carcinoembryonic antigen and advanced stage cancer ($p = 0.038$ and $p = 0.017$, respectively). CTC counts for patients with stage IV cancer (12.47 ± 24.00 per 5 ml, n = 15) were significantly higher than those for patients with cancers that were stage I-III (2.84 ± 5.29 per 5 ml, n = 61, $p = 0.005$) and healthy controls (0.25 ± 0.55 per 5 ml, n = 20, $p < 0.001$). In addition, progression-free survival tended to be lower in CTC-positive patients compared to CTC-negative patients ($p = 0.092$). In patients with stage I-III cancer, recurrence occurred only in CTC-positive patients.

Conclusion: Using this new size-based platform, CTC positivity was found to correlate with clinical features of colorectal cancer patients. Our results suggest that this new platform has potential for determining prognosis and therapeutic response in colorectal cancer patients.

Introduction

Cancer metastases occur when tumor cells derived from the primary tumor circulate in the peripheral blood and migrate to other organs [1, 2]. These cells are termed circulating tumor cells (CTCs), which are frequently detected in the blood and bone marrow of cancer patients [1]. The presence of CTCs is one of the steps necessary to develop distant metastases [2]. The characterization of CTCs may provide improved understanding of tumor metastasis biology. There are many reports on the clinical significance of CTCs for predicting the prognosis of patients [3-6]. In addition, CTCs may be useful for therapeutic response evaluation and the establishment of new therapeutic strategies.

Numerous technologies have been developed to detect and characterize CTCs from patient blood [7-10]. Among them, the CellSearch® system is the most commonly used and only FDA-approved platform for clinical use in metastatic breast, colorectal, or prostate cancer. This system uses magnetic beads that selectively bind to antibody for epithelial cell adhesion molecules (EpCAM) on CTCs [11, 12]. However, this system has a critical limitation in that it cannot detect CTCs undergoing epithelial-mesenchymal transition, which occurs due to the loss of epithelial markers such as EpCAM [13, 14]. In addition, this system cannot obtain viable cells that can be used for downstream analysis. New methods to address these problems have been developed. Accordingly, several size-based methods have been proposed that may be more efficient to isolate undetectable CTCs using the CellSearch® system [15]. Several studies have demonstrated that these size-based techniques were more sensitive than the CellSearch® system to detect CTCs in liver cancer, lung cancer, prostate cancer, and melanoma [16-19]. A recent study proposed a new technology for CTC isolation that is a size-based method using a Cytogen high-density microporous (HDM) chip [20]. However, no studies have investigated the efficacy of this platform in colorectal cancer, while

much effort has been directed to detect CTCs. In this study, we aimed to evaluate the clinical efficacy of this new size-based CTC detection platform in colorectal cancer.

Materials and methods

Patients and sample collection

This study was approved by the Samsung Medical Center Institutional Review Board (No. 2011-12-024) and written informed consent was obtained for all enrolled patients. We included 76 patients who were diagnosed with primary colorectal cancer and scheduled for surgery from October 2012 to December 2013 at the Samsung Medical Center. Patients were excluded if they had recurrent disease, synchronous malignancies, previous treatment for cancer, or underwent a local excision for a primary lesion. Peripheral blood samples (5 ml) were taken from the enrolled patients before surgery. Blood samples were also collected from 20 healthy volunteers as normal controls.

CTC enrichment

Blood samples from each patient were collected by venipuncture into Becton Dickinson vacutainer tubes containing Acid Citrate Dextrose Solution A (REF 364606, BD Franklin Lakes, NJ, USA), and were processed through the CTC isolation kit (Cat# CIKW10, Cytogen, Inc., Seoul, Republic of Korea). Blood samples were incubated for 20 min with an antibody cocktail against RBCs and WBCs and mixed with a preactivation buffer followed by density gradient centrifugation. The cell suspension containing CTCs was diluted with dilution buffer followed by filtering through a HDM chip [20]. Cells on the HDM chip were retrieved and

transferred to a microtube. Cell sizes were measured using an automatic cell counter (NanoEnTek). For immunofluorescent staining, isolated cells were fixed in 4% paraformaldehyde for 5 min at room temperature.

CTC identification

Enriched cells were recovered from the microporous filter and harvested on a microscope slide using Shandon Cytospin™ 4 (Thermo Fisher Scientific, Waltham, MA, USA). Cells on the microscope slides were fixed with 2% paraformaldehyde in PBS for 5 min and permeabilized with 0.2% Triton X-100 (Sigma-Aldrich, MO, USA) for 10 min. Fixed cells were blocked with 1% bovine serum albumin in PBS for 30 min at room temperature and incubated with 1:200 diluted anti-human EpCAM mAb (clone name VU1D9, #2929, Cell Signaling Technology, Danvers, MA, USA) for 1 h at room temperature. To enhance EpCAM signal, a tyramide signal amplification kit (MP 20911, Invitrogen, Carlsbad, CA, USA) was used. Cells were washed three times with PBS and incubated with 1:10 diluted anti-human CD45 mAb (clone name H230, sc-25590, Santa Cruz Biotechnology, Heidelberg, Germany) for 90 min at 37ºC. After washing 3 times with PBS, cells were incubated with Alexa Fluor 594-conjugated secondary Ab (1:300, Invitrogen) for 90 min at 37ºC. Cells were stained with FITC conjugated anti-cytokeratin (CK) and mounted with 4’,6-diamidino-2-phenylindole (DAPI) (MaxVision, Washington, USA) to visualize nuclei. CTCs were defined as DAPI-positive, CD45-negative, and EpCAM-positive cells or DAPI-positive, CD45-negative, and CK-positive cells.

Outcome assessments

All patients underwent radical surgery for a primary lesion and postoperative surveillance was recommended according to the protocols of our institution. Clinicopathologic features were evaluated and TNM stage was defined according to the Cancer Staging Manual Seventh Edition by the American Joint Committee on Cancer. CTCs were identified using immunofluorescent staining with DAPI, CD45, EpCAM, and CK, and defined as DAPI+/CD45-/EpCAM+ or CK+ cells. CTC positivity was determined when there were one or more CTCs. Clinical outcomes of patients were analyzed according to CTC counts.

Statistical analysis

Statistical analysis was performed using SPSS for Windows version 20.0 (IBM SPSS Statistics, IBM Corporation, Armonk, NY, USA). Descriptive statistics for clinical, imaging, and pathological variables were determined using mean and standard deviation or number with percent as appropriate. Patient characteristics according to CTC counts were compared using the chi-squared test or linear-by-linear association. Survival outcomes were analyzed using the Kaplan–Meier method and log-rank test. *P*-values were derived from two-tailed tests and $p < 0.05$ was considered statistically significant.

Results

Patient characteristics

Patient characteristics are shown in Table 1. Of the 76 patients, 40 were male and 36 were female, with a median age of 60 years (range, 32-86). Regarding pathological disease stage, 9 patients were stage I, 20 patients were stage II, 32 patients were stage III, and 15 patients

were stage IV. Lymphatic invasion and vascular invasion were noted in 21 and 19 patients, respectively. Thirty-seven patients received adjuvant chemotherapy after operation.

CTC counts according to clinicopathologic features

We counted CTCs in peripheral blood from enrolled patients (n = 76) using the four color staining protocol of DAPI for nucleated cells, CD45 mAb as a leukocyte marker, and EpCAM mAb or CK mAb as an epithelial cell marker. CTCs of epithelial origin were distinguished from blood cells using these markers. The results demonstrated that DAPI-positive, CD45-negative, and EpCAM-positive or CK-positive cells were counted (Fig. 1).

Of the 76 patients, CTCs were detected in 50 patients (65.8%). CTC-positive patients according to patient characteristics are presented in Table 2. CTC-positive patients were more frequently identified in patients with high level of carcinoembryonic antigen (CEA), which has been used as the typical marker for circulating tumor cells ($p = 0.038$). Advanced stage also significantly correlated with CTC positivity ($p = 0.017$). The percentages of CTC-positive samples per 5 ml blood according to cancer stage were 44.4% for stage I, 55.0% for stage II, 68.8% for stage III, and 86.7% for stage IV. Except for CEA level and cancer stage, there was no significant difference according to CTC positivity relative to other patient characteristics. In addition, CTC counts for patients with stage IV cancer (12.47 ± 24.00 per 5 ml, n = 15) were significantly higher than those for patients with cancers that were stage I-III (2.84 ± 5.29 per 5 ml, n = 61, $p = 0.005$) and healthy donors (0.25 ± 0.55 per 5 ml, n = 20, $p < 0.001$) (Table 3).

Survival outcomes according to CTC count

To better understand the relationship between CTC count and clinical outcomes, we analyzed the survival outcomes of patients according to CTC positivity. The median follow-up was 19.5 months, and there were 15 recurrences and 3 deaths. The mean progression-free survival (PFS) of CTC-positive patients tended to be lower than that of CTC-negative patients, but there was no statistical significance found (21.8 ± 1.40 months *vs.* 25.1 ± 1.13 months, p = 0.092) (Fig. 2A). All 3 patients who died during the follow-up period belonged to the CTC-positive group (Fig. 2B).

In addition, the clinical outcome of patients with stage I-III cancer was evaluated to assess potential role in predicting recurrence of patients who underwent radical surgery with curative intent. Of these 61 patients, 5 recurrences were observed. Surprisingly, these 5 patients belonged to the CTC-positive group and no recurrence was observed in the CTC-negative group (Table 4).

Discussion

In this study, we investigated the clinical efficacy of a cell size-based platform for CTC detection in colorectal cancer. Of 76 patients who underwent surgery for colorectal cancer, 50 were detected with one or more CTC. We found that as patient CEA level or cancer stage was higher, the CTC positivity seemed to be higher as well. In addition, PFS tended to be lower in CTC-positive patients compared to CTC-negative patients. Especially, recurrences occurred only in CTC-positive patients with stage I-III cancer. These findings suggest that this new size-based platform for CTC detection serves as a useful tool to predict prognosis and recurrence of colorectal cancer patients.

There are various diagnostic methods to improve the detection rate of CTCs [21-23]. However, many of them are not yet standardized and there are arguments in regards to usefulness of each method [23]. Until now, the CellSearch® system, which immunologically identifies EpCAM, is the most commonly used method in this field [11, 23]. However, the CellSearch® system cannot be used to identify EpCAM-negative cells such as CTCs undergoing the epithelial-mesenchymal transition process [13, 14] and a recent study reported that a significant portion of CTCs are EpCAM-negative [24]. In addition, the CellSearch® system is unable to detect viable cells because ferrofluid has dose-dependent cytotoxicity and magnetic beads suppress cell proliferation and metabolism [25]. Thus, this system cannot perform downstream analysis such as genomic analysis. Therefore, new technologies using a cell size-based system have emerged to overcome the disadvantages of the CellSearch® system [15, 17, 26]. A number of reports on size-based systems have been published since the first report in 2000 by Paterlini-Brechot et al. [27], which showed it might have an advantage over the CellSearch® system for capturing extremely rare CTCs in the blood because its enrichment principle is based on size rather than specific tumor antigens. It provides good sensitivity and specificity, simplicity, and low cost [17]. Especially, a new cell size-based platform using a HDM chip enables subsequent molecular characterization and analysis because the retrieved cells are viable and untouched. The microfabricated filter of the HDM chip has an optimized pore size and gap distance, and obtains sufficiently pure CTCs [20].

Since the concept of CTCs was introduced by Ashworth [28], their clinical significance has been emphasized and has been described in many studies [29, 30]. CTCs have potential roles in investigating the mechanism of metastasis, predicting cancer progression or metastasis, and evaluating treatment response [2, 15]. These clinical implications of CTCs in colorectal cancer have been described in several studies, where CTCs were more highly

expressed in cancer patients than in normal healthy subjects and CTC expression levels tend to be higher as cancer stage increases [4, 5, 31]. Recurrence and metastasis were more prevalent in patients who showed CTC expression and showed worse survival outcomes compared to patients without CTC expression [3, 4, 32]. Accordingly, monitoring CTC count in cancer patients over the disease course and period of treatment could provide guidelines for adequate management.

There are few standardized methods for detecting CTCs in colorectal cancer. The clinical efficacy of a new method using a HDM chip has already been validated in patients with non-small cell lung cancer (data not shown, but scheduled for publication). Thus, we subsequently performed a clinical validation study of 76 patients with colorectal cancer. To avoid chemotherapeutic interference with CTC enumeration, blood was drawn from patients before they received surgery or chemotherapy. The CTC count for patients with stage IV cancer was significantly higher than in patients with cancer at other stages and in normal healthy donors. In addition, PFS tended to be lower in the CTC-positive group than in the CTC-negative group. In analysis of the patients with stage I-III cancer, recurrence occurred only in the CTC-positive group. Our results suggest the clinical usefulness of this new platform in terms of real-time monitoring of disease course without invasive tissue biopsy.

There are some limitations to this platform. It is difficult to completely distinguish CTCs and leukocytes by their size. Thus, immunostaining of CD45 was performed to exclude leukocytes. In addition, this platform can lose CTCs during processing steps such as cell transfer and centrifugation. Cancer cells are typically heterogeneous in size and are deformable, which might allow them to pass through the pores of the HDM filters used in this platform. However, we determined that this platform worked well with blood spiked with cancer cells of fairly uniform size. Despite these limitations, this platform has the potential to

detect CTCs without EpCAM expression by the addition of CK staining to the protocol. Also, this platform enables additional cell culture and downstream analysis by separating viable cells, which is a limitation of the CellSearch® system due to the use of magnetic beads. In addition, this platform was successfully developed to have clinical benefit in colorectal cancer and the clinical relevance of this platform should be further characterized by extending studies to the genetic analysis of CTCs in the future.

In our study, this new size-based platform was first demonstrated for its clinical efficacy in colorectal cancer patients. It enables the detection of more cancer cells including those undergoing the epithelial-mesenchymal transition by identifying DAPI+/CD45-/EpCAM+ or CK+ cells. Our results suggest that this new platform has potential for future application in the real-time monitoring of cancer progression and therapeutic response.

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Conflicts of interest

None.

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CTC	DAPI	CD45	EpCAM	CK	Merged
DAPI+/CD45-/EpCAM +/CK-					
DAPI+/CD45-/EpCAM-/CK+					
DAPI+/CD45-/EpCAM +/CK+					
Leukocyte (DAPI+/CD45+/EpCAM-/CK-)					

Figure 1. Representative immunofluorescent images for different detected cell types in colorectal cancer patients. In order from above, DAPI+/CD45-/EpCAM+/CK- circulating tumor cells (CTCs), DAPI+/CD45-/EpCAM-/CK+ CTCs, DAPI+/CD45-/EpCAM+/CK+ CTCs, and DAPI+/CD45+/EpCAM-/CK- leukocytes.

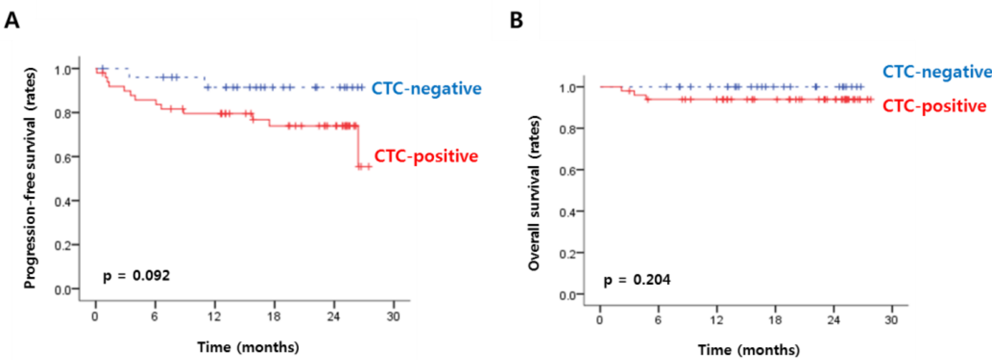

Figure 2. Kaplan-Meier survival plots of circulating tumor cell (CTC)-positive *vs*. CTC-negative patients. (A) Progression-free survival and (B) overall survival

Table 1. Patient characteristics

Characteristic	Number of patients
Total, n	76
Median age, years (range)	60.0 (32-86)
Gender, n (%)	
Male	40 (52.6%)
Female	36 (47.4%)
Preoperative CEA level, n (%)	
< 5 ng/ml	52 (68.4%)
≥ 5 ng/ml	24 (31.6%)
Location of primary tumor, n (%)	
Colon	50 (65.8%)
Rectum	26 (34.2%)
Stage, n (%)	
I	9 (11.9%)
II	20 (26.3%)
III	32 (42.1%)
IV	15 (19.7%)
Cell type, n (%)	
WD/MD	62 (81.6%)
PD/MUC	14 (18.4%)
Lymphatic invasion, n (%)	
Yes	21 (27.6%)
No	55 (72.4%)
Vascular invasion, n (%)	
Yes	19 (25.0%)
No	57 (75.0%)
Perineural invasion, n (%)	
Yes	26 (34.2%)
No	50 (65.8%)
Chemotherapy, n (%)	
Yes	37 (48.7%)
No	39 (51.3%)

CEA, carcinoembryonic antigen; WD, well differentiated; MD, moderately differentiated; PD, poorly differentiated; MUC, mucinous carcinoma.

Table 2. Circulating tumor cells according to patient characteristics

Characteristics	CTC-positive patients	P -value
Age, years (%)		0.833
< 65 (n = 48)	32 (66.7%)	
≥ 65 (n = 28)	18 (64.3%)	
Gender, n (%)		0.740
Male (n = 40)	27 (67.5%)	
Female (n = 36)	23 (63.9%)	
Preoperative CEA level, n (%)		0.038
< 5 ng/ml (n = 52)	30 (57.7%)	
≥ 5 ng/ml (n = 24)	20 (83.3%)	
Location of tumor, n (%)		0.334
Colon (n = 50)	31 (62.0%)	
Rectum (n = 26)	19 (73.1%)	
Stage, n (%)		0.017
I (n = 9)	4 (44.4%)	
II (n = 20)	11 (55.0%)	
III (n = 32)	22 (68.8%)	
IV (n= 15)	13 (86.7%)	
Cell type, n (%)		0.357
WD/MD (n = 62)	39 (62.9%)	
PD/MUC (n = 14)	11 (78.6%)	
Lymphatic invasion, n (%)		0.238
Yes (n = 21)	16 (76.2%)	
No (n = 55)	34 (61.8%)	
Vascular invasion, n (%)		0.264
Yes (n = 19)	15 (78.9%)	
No (n = 57)	35 (61.4%)	
Perineural invasion, n (%)		0.334
Yes (n = 26)	19 (73.1%)	
No (n = 50)	31 (62.0%)	

CTC, circulating tumor cell; CEA, carcinoembryonic antigen; WD, well differentiated; MD,

moderately differentiated; PD, poorly differentiated; MUC, mucinous carcinoma.

Table 3. Number of circulating tumor cells according to stage

CTC number	Stage I-III (n = 61)	Stage IV (n = 15)	Control group (n = 20)
0	24 (39.3%)	2 (13.3%)	16 (80.0%)
1	14 (23.0%)	4 (26.7%)	3 (15.0%)
2	8 (13.1%)	3 (20.0%)	1 (5.0%)
3	2 (3.3%)	1 (6.7%)	0 (0.0%)
4	3 (4.9%)	0 (0.0%)	0 (0.0%)
5	0 (0.0%)	0 (0.0%)	0 (0.0%)
> 5	10 (16.4%)	5 (33.3%)	0 (0.0%)
Mean ± SD	2.84 ± 5.29	12.47 ± 24.00	0.25 ± 0.55

CTC, circulating tumor cell; SD, standard deviation.

Table 4. Number of recurrences according to stage and CTC-positivity

	Stage I-III (n = 61)	Stage IV (n = 15)
Total recurrences, n	5	10
CTC-positive patients	5	8
CTC-negative patients	0	2

CTC, circulating tumor cell.