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Original Articles

Cytokeratin 20 improves the detection of circulating tumor cells in patients with colorectal cancer

Charlotte Welinder [a], Bo Jansson [a], Gert Lindell [b], Jörgen Wenner [c,*]

[a] Department of Oncology, Clinical Sciences, Lund University, SE-221 85 Lund, Sweden
[b] Hepato-Biliary and Pancreatic Surgery, Department of Surgery, Skane University Hospital (SUS), Lund, Sweden
[c] Department of Surgery and Urology, Office for Health Care Sund, Helsingborg Hospital, SE-251 87 Helsingborg, Sweden

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ABSTRACT

Cytokeratin 20 (CK20) is a well-established marker for colon epithelium. Herein, we suggest that CK20 is a biomarker for detecting circulating tumor cells (CTCs) in patients with metastatic colorectal cancer. Blood specimens (7.5 mL) were collected during surgery after liver mobilization from 25 patients with colorectal cancer. The FDA approved CellSearch™ system and two panels of antibodies against cytokeratins, cytokeratin 8, 18 and 19 (CK8/18/19) and CK8/18/19/20, were used for the detection of CTCs. All the patients' samples were processed using the anti-CK8/18/19 panel. The number of detected CTCs was low, 52% of the patients lacked CTCs and 40% had ≤ 2 CTCs/7.5 mL blood. Nine of the patients' blood samples were processed with both antibody panels. The detection rate of CTCs was significantly higher using the anti-CK8/18/19/20 panel compared with the anti-CK8/18/19 panel, *p*-value 0.0078. Our data show that inclusion of CK20 as a biomarker efficiently improves the detection of CTCs in colorectal cancer patients. The finding in our study is of clinical importance since a new prognostic biomarker would provide an important tool in individual clinical decision-making for colorectal cancer patients.

Introduction

Colorectal cancer (CRC) is the third most frequent cancer with 6000 new cases per year in Sweden [1] and 1.2 million per year worldwide [2]. Approximately 50% of patients with CRC develop liver metastases (LM) and 15–20% of these patients will be candidates for curative liver resection [3]. Long-term outcome after liver resection for CRC-LM has improved over time and an overall 5-year survival of 45–50% can be expected in combination with modern chemotherapy and targeted drugs [4,5]. Despite improved overall survival, recurrence rates have remained relatively unchanged and more than 50% of patients will develop recurrence within 2 years after liver resection [6]. For patients with metastatic colorectal cancer the presence of circulating tumor cells (CTCs) has been reported as an independent predictor of overall and progression-free survival [7]. The risks associated with tumor manipulation during surgery and exact mechanisms involved in the development of recurrent metastases are unknown and CTCs can be an important way to monitor tumor cell spread during liver surgery [8].

In year 2004, the CellSearch™ system (Veridex LCC, Raritan, NJ, USA) was cleared by the US Food and Drug Administration as a diagnostic tool for identifying and counting CTCs in blood samples in patients with metastatic breast cancer. Later, 2007 and 2008, FDA also cleared the system for monitoring CTCs in patients with colon cancer and prostate cancer, respectively.

Numerous studies of patients with both advanced colorectal cancer and non-metastatic colorectal cancer have shown that the number of detectable CTCs using the CellSearch™ system is low and even below the clinical threshold of three CTCs, which is in contrast to other epithelial tumor types e.g. breast and prostate cancer [9–17].

Attempts have been made to improve CellSearch based CTC detection in patients with colorectal cancer using larger sample volumes, 30 mL blood instead of 7.5 mL [14,18]. The study by Lalmahomed [14], including 15 patients with colorectal liver metastases, showed that the number of CTCs was significantly higher using 30 mL instead of 7.5 mL blood. Before the CellSearch analyses the 30 mL blood was reduced to a volume of 7.5 mL enriched blood using modified Ficoll density gradient separation. Analyzing the enriched blood ≥1 CTC was found in 13 patients (87%) and ≥3 CTCs were found in seven patients (47%) compared with ≥1 CTC in 10 patients (67%) and ≥3 CTCs in two patients (13%) analyzing 7.5 mL blood.

Gervasoni et al. [19] compared three distinct methods for the detection of CTCs in colorectal cancer patients: a multimarker RT-PCR assay, the CellSearch™ system and dHPLC-based gene mutation analysis. The study included 20 patients undergoing elective surgery for colorectal adenocarcinoma and 75% of the patients were

* Corresponding author. Tel.: +46 42 4062904; fax: +46 42 4061516.
E-mail address: jorgen.wenner@skane.se (J. Wenner).

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positive for the presence of CTCs by the multimarker RT-PCR method. The multimarker RT-PCR assay included expression analysis of four genes, carcinoembryonic antigen (CEA), CK20, CK19 and guanylyl cyclase C. Only 20% were positive by the CellSearch™ system and 14.3% of the patients displayed gene mutations when the dHPLC method was applied.

Cytokeratin 20 expression is a marker for colorectal adenocarcinomas with clinical immunohistochemistry [20]. In this study we wanted to investigate anti-CK20 as an additional marker for CTCs in blood from colorectal cancer patients using the CellSearch™ system, in order to detect those cancer cells that lack the expression of CK 8/18/19.

Materials and methods

Patients

Patients planned for liver resection of colorectal liver metastases at the Department of Surgery, Skåne University Hospital, were asked to participate in the study. Written informed consent was obtained from all participants. The Ethics Committee of Lund University approved the study (LU 2009-746).

Blood samples

The peripheral blood samples (7.5 mL) were drawn from each patient through direct puncture of the cephalic vein after mobilization of the liver and transection of the parenchyma. All samples were taken in CellSave® preservative tubes (Veridex, Warren, NJ, USA), maintained at room temperature and processed within 72 hours.

Sample processing

The Cell Search® technology has been previously described in detail [9]. In summary, the Cell Search® System is a semi-automated technique used to enrich and enumerate CTCs in patient blood samples. Using the blood from the CellSave® (Veridex) preservative tubes, 7.5 mL was mixed with 6.5 mL dilution buffer and centrifuged at 800 × *g* for 10 min at room temperature. The centrifuged samples were transferred to the CellTracks®AutoPrep® system (Veridex) where the blood samples were automatically processed. The CTCs were immunomagnetically enriched and stained using the CellSearch CTC Test kit. The CTC kit contains: anti-epithelial cell adhesion molecule (EpCAM) antibody conjugated with ferrofluid which immunomagnetically enriches the EpCAM positive CTCs; staining reagents diamino-2-phenylindole (DAPI) which stain the nuclei; antibodies against CD45 conjugated with allophycocyanin (APC) for leucocytes exclusion; and antibodies against cytokeratins 8, 18 and 19 conjugated with phycoerythrin (PE) for positive detection of CTC. Cytokeratin 20 expression of CTCs was characterized within the Cell Search® System by the addition of PE-labeled anti-cytokeratin 20 antibody (working solution 2 μg/mL). After enrichment and staining of cells, the sample was dispended into a cartridge in a MagNest® (Veridex) and analyzed with the CellTracks® Analyser II (Veridex). The CellTracks® Analyser II is a semiautomatic microscope, which preanalyzes CTC captured by the CellTracks®AutoPrep® system and shows stained cells to trained operators who decide the presence of CTC in the samples. Cytokeratin positivity, positive nuclear staining and CD45 negativity identify the CTCs. Two independent certified operators analyzed and verified each patient sample in an operator-blinded fashion, and disagreements were solved by discussion.

Cell lines

The human colon carcinoma cell lines, Lovo, Colo205 and CaCO2, breast cancer cell lines T47D, MCF7 and the Burkit lymphoma cell line Raji were all purchased from the ATCC (Rockville, MD, USA) and cultured in RPMI 1640 (Gibco®, Life Technologies) supplied with 10% fetal bovine serum.

Labeling of antibody

The CK20 specific monoclonal antibody, Ks 20.8 (cat no 61026, PROGEN Biotechnik, Heidelberg, Germany) was labeled with LYNX Rapid RPE antibody conjugation kit (AbD Serotec, Oxford, UK) according to manufacturer's instruction, using 100 μg of antibody and 50 μg of labeling regents and overnight incubation.

Flow cytometry

The labeled antibody was tested in flow cytometry. Cell lines were fixed and permeabilized using Cellsearch standard reagents and anti-CK20 antibody labeled with RPE was diluted to a final volume of 1 μg/mL and incubated with different cell lines for 1 h in room temperature before being analyzed using a FACSAria (BD Bioscience). Data were evaluated by Flowing software (Turku Centre for Biotechnology, Turku, Finland).

Table 1
Patient demographics and clinical characteristics ($n = 25$).

	n
Age	73 [51–81]
Gender, men/women	16/9
Primary tumor location	
Colon	18 (72%)
Rectum	7 (28%)
Primary tumor in situ ("liver first")	6
T stage	
T2	1
T3	13
T4	5
N stage	
N0	8
N1	6
N2	5
Vascular/perineural invasion	5/7
Synchronous/metachronous LM	16/9
Bilobar LM	6 (24%)
Number of LM	2 [1–6]
Largest LM, mm	35 [10–140]
Chemotherapy within 3 months	16 (64%)

Median [range].

Statistical analysis

For statistical analysis a Wilcoxon matched-pairs signed rank test was used (Prism v6.04, GraphPad Software, Inc.).

Results

Patient characteristics

Twenty-five patients accepted to participate in the study. Sixteen patients (64%) received combination chemotherapy within 3 months prior to liver resection. Five patients received irinotecan-based therapy with additional monoclonal antibodies in four of these patients. Nine patients had oxaliplatin-based treatment in combination with cetuximab in two of these patients. One patient received both oxaliplatin and irinotecan prior to liver surgery. Four patients did not receive any chemotherapy. Chemotherapy was routinely terminated 2–4 weeks before liver resection, and a free interval of 6 weeks was used after treatment with bevazicumab.

Patient demographics and clinical characteristics are shown in Table 1. The median time from diagnosis of the colorectal cancer to diagnosis of LM was 12 months (range, 5–16 months) for patients with metachronous disease.

Detection of CTCs using CellSearch™ system with the anti-CK8/18/19 panel

A total of 25 blood samples from metastatic colorectal cancer were tested using the anti-CK8/18/19 panel and the number of detected tumors cells during liver resection are shown in Table 2. Circulating tumor cells was detected in 48% of the patients. However, the number of detected CTC was generally low often below the clinical threshold of three CTCs using the anti-CK8/18/19 panel. Thirteen patients lacked CTCs, eight patients had one CTC, and four

Table 2
Circulating tumor cells (CTC) in peripheral blood samples in patients undergoing liver resection for colorectal cancer liver metastasis ($n = 25$) using standard kit with CK8/18/19.

No. of patients with 0 CTC	13 (52%)
No. of patients with 1–2 CTCs	10 (40%)
No. of patients with CTC ≥3	2 (8%)
No. of detected CTC, median [range]	7 [0–162]