Low Number of Detectable Circulating Tumor Cells in Non-metastatic Colon Cancer

MORTEN THORSTEINSSON[1], GYÖRGY SÖLETORMOS[2] and PER JESS[1]

[1]*Department of Surgery, Roskilde Hospital, University of Copenhagen, DK-4000 Roskilde, Denmark;*
[2]*Department of Clinical Biochemistry, Hillerœd Hospital, University of Copenhagen, DK-3400 Hilleroed, Denmark*

Abstract. *Background: The aim of the present study was to detect circulating tumor cells (CTCs) in the peripheral blood of patients with non-metastatic colon cancer and to evaluate whether there is a diurnal variation in the CTC counts. Furthermore, the study aimed to examine the correlation between CTCs and TNM stage, other paraclinical variables and prognosis. Patients and Methods: Blood samples were collected from 20 consecutive patients with colon cancer TNM stage I-III at four different perioperative time points. Detection of CTCs was performed using the immunological assay CellSearch®. Results: CTCs were detected in 1 out of 60 preoperative blood samples, resulting in a detection rate of 1 in 20 patients (5%; 95% confidence interval=0.1-25%). None of the postoperative blood samples had CTC levels above the cut-off value (≥2 CTCs/7.5ml blood). Conclusion: The presence of CTCs in non-metastatic colon cancer is rare and barely detectable with the only commercially available assay for detection of CTCs, the CellSearch System.*

The presence of circulating tumor cells (CTCs) in the peripheral blood of patients with metastatic breast, prostate and colorectal cancer has proven to be a strong prognostic marker (1-4). Two prospective studies have evaluated CTCs in patients with metastatic colorectal cancer and demonstrated that the presence of CTCs was an independent predictor of decreased progression-free survival and overall survival (1, 4). These studies used the only commercially available assay for detection of CTC, VERIDEX, the CellSearch®, Circulating Tumor Cell Test (LLC). This semiautomated immunological technique has been approved by the US Food and Drug Administration for the detection

of CTCs in metastatic breast, prostate and colorectal cancer. Allard *et al.* (5) evaluated the CellSearch System and established a cut-off value of ≥2 CTCs per 7.5 ml peripheral blood in patients with various metastatic carcinomas. With this method, CTCs were detectable in approximately 30% of patients with metastatic colorectal cancer (1, 4).

Only a few studies have utilized the CellSearch System in non-metastatic colorectal cancer. Sastre *et al.* (6) demonstrated that CTCs were detected in 26% of patients tested postoperatively, but two other studies reported lower detection rates, 7% of patients tested preoperatively (7) and 15% of patients tested postoperatively with the Cellsearch assay (8). There are no data on the prognostic significance of CTCs in non-metastatic colorectal cancer applying the CellSearch assay.

With reference to these results a study was conducted to determine the presence of CTCs pre- and postoperatively in patients with non-metastatic colon cancer. The aims were: to evaluate whether there was a diurnal variation of CTCs preoperatively, to correlate the presence of CTCs with TNM-stage and other paraclinical variables and to determine whether the postoperative presence of CTCs predicts recurrence.

Patients and Methods

Patients. The prospective study included 20 consecutive patients with non-metastatic colon cancer, who underwent curative colon resection at the Department of Surgery, Roskilde Hospital, Denmark between February and July 2010. The study was approved by the Danish National Committee on Biomedical Research Ethics. The inclusion criteria were signed informed consent, newly diagnosed and histologically verified adenocarcinoma of the colon (TNM stage I-III), absence of other malignant diseases and no prior chemo- or radiotherapy. All the patients were preoperatively assessed with a computed tomography (CT) scan of the thorax and abdomen. Patients with distant metastases (TNM stage IV) were excluded. The tumors were classified according to the fifth edition of the TNM classification of the International Union Against Cancer (9). The patient characteristics such as age, gender, tumor location, TNM stage, histological differentiation, venous invasion, lymph node metastasis and carcinoembryonic antigen (CEA) level were recorded (Table I).

Correspondence to: Morten Thorsteinsson, MD, Department of Surgery, Roskilde Hospital, University of Copenhagen, Koegevej 7-13, DK-4000 Roskilde, Denmark, Tel: +45 61276425, Fax: +45 47323009, e-mail: moth@regionsjaelland.dk

Table I. *Patient characteristics*.

Characteristics	All patients (n=20)
Age, median (range), years	70 (44-86)
Gender	
Male	12 (60%)
Female	8 (40%)
Tumor location	
Right colon	9 (45%)
Transverse colon	2 (10%)
Left colon	0
Sigmoid colon	9 (45%)
TNM stage	
I	3 (15%)
II	8 (40%)
III	9 (45%)
Differentiation	
Well	9 (45%)
Moderate	7 (35%)
Poor	3 (15%)
Unknown	1 (5%)
Venous invasion	
No	18 (90%)
Yes	2 (10%)
Lymph node metastasis	
No	12 (60%)
Yes	8 (40%)
Preoperative CEA level	
<5 ng/ml	17 (85%)
>5 ng/ml	3 (15%)

Blood samples. Peripheral blood was sampled *via* an intravenous cannula on four separate occasions: three times preoperatively and once postoperatively. The first two blood samples were collected the day before surgery at 8 am and 8 pm, and the third blood sample was drawn at 8 am on the day of surgery. The fourth blood sample was drawn 30 days after surgery and before starting adjuvant chemotherapy.

CTC detection. All blood samples were collected into 10 ml CellSave® Preservative Tubes (Veridex, LLC). The samples were maintained at room temperature and processed within 96 hours after collection according to the guidelines at the manufacturer. The analysis was blinded and conducted by trained specialists at Source BioScience, Nottingham, UK, without any information concerning the clinical characteristics of the patients.

For isolation and enumeration of CTCs, the Cellsearch® Circulating Tumor Cell Test (Veridex, LLC) was used. This assay consists of a semiautomated system where cells expressing epithelial-cell adhesion molecule (EpCAM) on their cell membranes are enriched by immunomagnetic separation using ferro-fluids coated with EpCAM antibodies. The isolated cells were fluorescently labeled with nucleic acid dye 4,2-diamidino-2-phenylindole (DAPI) and monoclonal antibodies against cytokeratin 8, 18 and 19. Monoclonal antibodies against cluster of differentiation 45 antigen (CD45, a common leucocyte antigen) were used to distinguish epithelial cells from leucocytes. Further

identification and enumeration of CTCs was performed by the CellTracks® Analyzer II, a semiautomated fluorescence microscope. Finally a gallery of cellular images was reviewed by a trained specialist who made the ultimate selection of CTCs. CTCs were defined as nucleated cells (DAPI-positive) staining positive for cytokeratin and negative for CD45, and showing cellular morphology. The results were expressed as the number of CTCs per 7.5 ml peripheral blood. The cut-off value for a positive result was chosen to be ≥2 CTCs per 7.5 ml blood (5).

Statistical analysis. The Chi-square test was used to compare the present results with findings of other studies. The level of significance was set at $p<0.05$ (two-sided). All the statistical calculations were carried out using SAS version 9.1 for Windows (SAS Institute, Cary, NC, USA).

Results

Twenty patients were included in the study, 12 males and 8 females, with a median age of 70 years (range 44-86). The patient characteristics are summarized in Table I. One patient died of respiratory complications 11 days after surgery and was excluded from the postoperative 30 days' follow-up. Two patients underwent open surgery and the remaining 18 were operated laparoscopically. All the tumors were adenocarcinomas. Three patients were diagnosed with TNM stage I, 8 patients with TNM stage II and 9 patients with TNM stage III. Preoperative CEA levels were elevated (>5 ng/ml, range 9-110 ng/ml) in three patients.

A total of 79 blood samples were collected and no samples were rejected due to processing errors. Only 1 out of the 60 preoperative blood samples had ≥2 CTCs/7.5 ml blood (1.7%; 95% CI=0.04-9%), resulting in a detection rate of 1 in 20 patients (5%; 95% CI=0.1-25%) (Table II). None of the 19 postoperative blood samples had CTC levels above the cut-off value (0%; 95% CI=0-18%). One postoperative blood sample was positive for 1 CTC/7.5 ml blood. Given the low CTC yield, there was no statistical power to determine diurnal variation, correlation between CTCs and paraclinical variables or impact on prognosis.

Discussion

The most widely used method of detecting CTCs in non-metastatic colorectal cancer is reverse-transcription polymerase chain reaction (RT-PCR) (10-14). Uen *et al*. (10) used a multi-marker RT-PCR method and found detectable CTCs in 137 out of 438 patients (31%) with TNM stage I-III colorectal cancer. This result was supported by Sadahiro *et al*. (11) who also used RT-PCR to detect CTCs in 44 out of 200 patients (22%) with TNM stage I-III colorectal cancer. Other RT-PCR studies have demonstrated lower detection rates of 4-6% (13, 14). These studies are difficult to compare because of heterogeneous study designs, especially differences in laboratory techniques and choice of marker

Table II. *Circulating tumor cell counts and clinical data.*

Patient no.	Age (years)	Gender	TNM stage	Preoperative CEA level ng/ml	CTC count sample			
					1	2	3	4
1	44	M	III	9.6	0	0	0	0
2	73	F	II	2.3	0	0	0	0
3	59	F	III	1.4	0	0	0	0
4	62	M	III	3.9	0	0	0	0
5	70	M	II	13.9	0	0	0	0
6	85	M	II	1.9	4	0	0	-
7	58	F	III	1.8	0	0	0	0
8	64	M	III	0.6	0	0	0	0
9	48	M	I	1.1	0	0	0	1
10	82	M	I	3.6	0	0	0	0
11	82	F	II	3.1	0	0	0	0
12	60	M	III	3.9	0	0	0	0
13	71	M	II	0.9	0	0	0	0
14	78	M	II	2.0	0	0	0	0
15	81	F	I	1.9	0	0	0	0
16	69	F	III	1.5	0	0	0	0
17	86	F	II	110.2	0	0	0	0
18	77	M	III	1.4	0	0	0	0
19	85	M	II	3.1	0	0	0	0
20	64	F	III	4.2	0	0	0	0

genes. Generally, the RT-PCR method is considered to have a higher sensitivity than the immunological techniques, but the specificity of RT-PCR may be hampered by high numbers of false-positive results due to contamination or target genes expressed in non-malignant cells (15-18). Therefore, the CellSearch System, a commercially available immunological technique that has been validated and found reproducible (1, 4-6, 19, 20) was chosen for the present CTC measurements.

In the present study, CTCs were found in only one preoperative sample (5%) (Table II), in accordance with the findings of Wind *et al*. (7), who found CTCs preoperatively in 2 out of 31 patients (7%) with non-metastatic colorectal cancer.

In the 19 postoperative samples, no sample with CTCs above the cut-off value was found. One sample was positive for 1 CTC/7.5 ml peripheral blood, but according to Allard *et al*. (5) this can be present in healthy individuals. The result was significantly different from that of Sastre *et al*. (6) who identified CTCs postoperatively in 17 out of 66 patients (26%) with TNM stage I-III colorectal cancer ($p<0.05$, Chi-square test). The differences can probably not be explained by the small sample size in the current study, but rather by laboratory differences, such as variation in the subjective verification of CTCs. In the study by Maestro *et al*. (8), CTCs were detected in 25 out of 164 patients (15%) with non-metastatic colorectal cancer, which was not significantly different from the present result ($p>0.05$, Chi-square test).

To overcome the inability to detect CTCs, Lalmahomed *et al*. (21) have recently described a method to improve the

sensitivity of the CellSearch System. The authors increased the blood sample size from 7.5 ml to 30 ml in 15 patients with colorectal liver metastases. The 30 ml of blood where thereafter reduced to a volume of 7.5 ml of enriched blood by density gradient separation. The median number of detected CTCs increased from 1 (0-4) in 7.5 ml blood to 2 (0-9) in 30 ml blood. In the current study, the preoperative blood sample size was increased to 30 ml (10 ml peripheral blood collected on three different occasions, within two days). Although the present results suggest otherwise, it is possible that the procedure described by Lalmahomed *et al*. (21) might also increase the CTC yield in patients with non-metastatic colorectal cancer.

More sensitive assays are needed to increase the percentages of detectable CTCs. Wong *et al*. (22) have developed a different immunological approach using a gastrointestinal-specific anti-cytokeratin 20 antibody to detect CTCs. They were able to demonstrate CTCs preoperatively in 58 out of 101 patients (57%) with stage I-III colorectal cancer and furthermore to detect a decrease in CTCs in 51 of these 58 patients (88%) after surgery (22).

The release of cancer cells from the primary tumor or metastases to the circulation is called shedding. It is unknown whether this process is continuous or dependent on a circadian rhythm. To our knowledge, no data exist on the diurnal variation of CTCs in non-metastatic colorectal cancer. In the current study, it was not possible to demonstrate a significant diurnal variation of CTCs because

of the low number of detectable CTCs. Tentatively, the preoperative sampling period may have been too short. Interestingly, intra-patient variation was found in the CTC counts. As demonstrated in Table II, the first preoperative blood sample of patient six was positive for CTCs, whereas the latter two were negative. The results suggest that CTCs in non-metastatic colon cancer are rare and that negative findings occur in repeated sampling with the applied method. In another study on patients with metastatic breast cancer, no significant differences between day-time and night-time CTC counts were found using the CellSearch method (23).

The percentage of patients with detectable CTCs increases with advancing TNM stage, as has been documented in several studies (6, 8, 22). Because of the low CTC yield in this study, a correlation between CTC and TNM stage, other paraclinical variables, and the potential impact on prognosis in non-metastatic colon cancer, could not be demonstrated.

In conclusion, the 'true' percentage of detectable CTCs with the current immunological assay remains unclear, but given the low CTC yield in non-metastatic colon cancer with the present assay, it seems unlikely that this approach can be used in a clinical setting to predict patients at high risk of recurrence. More sensitive assays or larger volumes of blood may increase the detection rate of CTCs. Thus, further studies primarily directed at improving the CTC detection methods are warranted before the clinical value of CTCs in non-metastatic colon cancer can be truly evaluated.

Acknowledgements

References

1 Cohen SJ, Punt CJ, Iannotti N, Saidman BH, Sabbath KD, Gabrail NY, Picus J, Morse M, Mitchell E, Miller MC, Doyle GV, Tissing H, Terstappen LW and Meropol NJ: Relationship of circulating tumor cells to tumor response, progression-free survival, and overall survival in patients with metastatic colorectal cancer. J Clin Oncol 26(19): 3213-3221, 2008.

2 de Bono JS, Scher HI, Montgomery RB, Parker C, Miller MC, Tissing H, Doyle GV, Terstappen LW, Pienta KJ and Raghavan D: Circulating tumor cells predict survival benefit from treatment in metastatic castration-resistant prostate cancer. Clin Cancer Res 14(19): 6302-6309, 2008.

3 Hayes DF, Cristofanilli M, Budd GT, Ellis MJ, Stopeck A, Miller MC, Matera J, Allard WJ, Doyle GV and Terstappen LW: Circulating tumor cells at each follow-up time point during therapy of metastatic breast cancer patients predict progression-free and overall survival. Clin Cancer Res 12(14 Pt 1): 4218-4224, 2006.

4 Tol J, Koopman M, Miller MC, Tibbe A, Cats A, Creemers GJ, Vos AH, Nagtegaal ID, Terstappen LW and Punt CJ: Circulating tumour cells early predict progression-free and overall survival in advanced colorectal cancer patients treated with chemotherapy and targeted agents. Ann Oncol 21(5): 1006-1012, 2010.

5 Allard WJ, Matera J, Miller MC, Repollet M, Connelly MC, Rao C, Tibbe AG, Uhr JW and Terstappen LW: Tumor cells circulate in the peripheral blood of all major carcinomas but not in healthy subjects or patients with nonmalignant diseases. Clin Cancer Res 10(20): 6897-6904, 2004.

6 Sastre J, Maestro ML, Puente J, Veganzones S, Alfonso R, Rafael S, Garcia-Saenz JA, Vidaurreta M, Martin M, Arroyo M, Sanz-Casla MT and Diaz-Rubio E: Circulating tumor cells in colorectal cancer: correlation with clinical and pathological variables. Ann Oncol 19(5): 935-938, 2008.

7 Wind J, Tuynman JB, Tibbe AG, Swennenhuis JF, Richel DJ, van Berge Henegouwen MI and Bemelman WA: Circulating tumour cells during laparoscopic and open surgery for primary colonic cancer in portal and peripheral blood. Eur J Surg Oncol 35(9): 942-950, 2009.

8 Maestro LM, Sastre J, Rafael SB, Veganzones SB, Vidaurreta M, Martin M, Olivier C, DE La Orden VB, Garcia-Saenz JA, Alfonso R, Arroyo M and Diaz-Rubio E: Circulating tumor cells in solid tumor in metastatic and localized stages. Anticancer Res 29(11): 4839-4843, 2009.

9 Sobin LH and Wittekind Ch, International Union Against Cancer (UICC). TNM Classification of Malignant Tumors. 5th ed. New York: John Wiley & Sons, Inc., 1997.

10 Uen YH, Lu CY, Tsai HL, Yu FJ, Huang MY, Cheng TL, Lin SR and Wang JY: Persistent presence of postoperative circulating tumor cells is a poor prognostic factor for patients with stage I-III colorectal cancer after curative resection. Ann Surg Oncol 15(8): 2120-2128, 2008.

11 Sadahiro S, Suzuki T, Maeda Y, Yurimoto S, Yasuda S, Makuuchi H, Kamijo A and Murayama C: Detection of carcinoembryonic antigen messenger RNA-expressing cells in peripheral blood 7 days after curative surgery is a novel prognostic factor in colorectal cancer. Ann Surg Oncol 14(3): 1092-1098, 2007.

12 Allen-Mersh TG, McCullough TK, Patel H, Wharton RQ, Glover C and Jonas SK: Role of circulating tumour cells in predicting recurrence after excision of primary colorectal carcinoma. Br J Surg 94(1): 96-105, 2007.

13 Iinuma H, Okinaga K, Egami H, Mimori K, Hayashi N, Nishida K, Adachi M, Mori M and Sasako M: Usefulness and clinical significance of quantitative real-time RT-PCR to detect isolated tumor cells in the peripheral blood and tumor drainage blood of patients with colorectal cancer. Int J Oncol 28(2): 297-306, 2006.

14 Lloyd JM, McIver CM, Stephenson SA, Hewett PJ, Rieger N and Hardingham JE: Identification of early-stage colorectal cancer patients at risk of relapse post-resection by immunobead reverse transcription-PCR analysis of peritoneal lavage fluid for malignant cells. Clin Cancer Res 12(2): 417-423, 2006.

15 Dandachi N, Balic M, Stanzer S, Halm M, Resel M, Hinterleitner TA, Samonigg H and Bauernhofer T: Critical evaluation of real-time reverse transcriptase-polymerase chain reaction for the quantitative detection of cytokeratin 20 mRNA in colorectal cancer patients. J Mol Diagn 7(5): 631-637, 2005.

16 Lambrechts AC, Bosma AJ, Klaver SG, Top B, Perebolte L, Veer LJ and Rodenhuis S: Comparison of immunocytochemistry, reverse transcriptase polymerase chain reaction, and nucleic acid sequence-based amplification for the detection of circulating breast cancer cells. Breast Cancer Res Treat 56(3): 219-231, 1999.

17 Novaes M, Bendit I, Garicochea B and del Giglio A: Reverse transcriptase-polymerase chain reaction analysis of cytokeratin 19 expression in the peripheral blood mononuclear cells of normal female blood donors. Mol Pathol *50(4)*: 209-211, 1997.

18 Smith BM, Slade MJ, English J, Graham H, Luchtenborg M, Sinnett HD, Cross NC and Coombes RC: Response of circulating tumor cells to systemic therapy in patients with metastatic breast cancer: comparison of quantitative polymerase chain reaction and immunocytochemical techniques. J Clin Oncol *18(7)*: 1432-1439, 2000.

19 Cohen SJ, Alpaugh RK, Gross S, O'Hara SM, Smirnov DA, Terstappen LW, Allard WJ, Bilbee M, Cheng JD, Hoffman JP, Lewis NL, Pellegrino A, Rogatko A, Sigurdson E, Wang H, Watson JC, Weiner LM and Meropol NJ: Isolation and characterization of circulating tumor cells in patients with metastatic colorectal cancer. Clin Colorectal Cancer *6(2)*:125-132, 2006.

20 Riethdorf S, Fritsche H, Muller V, Rau T, Schindlbeck C, Rack B, Janni W, Coith C, Beck K, Janicke F, Jackson S, Gornet T, Cristofanilli M and Pantel K: Detection of circulating tumor cells in peripheral blood of patients with metastatic breast cancer: a validation study of the CellSearch system. Clin Cancer Res *13(3)*: 920-928, 2007.

21 Lalmahomed ZS, Kraan J, Gratama JW, Mostert B, Sleijfer S and Verhoef C: Circulating tumor cells and sample size: the more, the better. J Clin Oncol *28(17)*: 288-289, 2010.

22 Wong SC, Chan CM, Ma BB, Hui EP, Ng SS, Lai PB, Cheung MT, Lo ES, Chan AK, Lam MY, Au TC and Chan AT: Clinical significance of cytokeratin 20-positive circulating tumor cells detected by a refined immunomagnetic enrichment assay in colorectal cancer patients. Clin Cancer Res *15(3)*: 1005-1012, 2009.

23 Martin M, Garcia-Saenz JA, Maestro De las Casas ML, Vidaurreta M, Puente J, Veganzones S, Rodriguez-Lajusticia L, De la Orden V, Oliva B, De la Torre JC, Lopez-Tarruella S, Casado A, Sastre J and Diaz-Rubio E: Circulating tumor cells in metastatic breast cancer: timing of blood extraction for analysis. Anticancer Res *29(10)*: 4185-4187, 2009.