

Monitoring of circulating epithelial tumor cells using the Maintrac[®] method and its potential benefit for the treatment of patients with colorectal cancer

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Abstract. Circulating tumor cells are an important link between primary tumors and metastases. A longitudinal monitoring of their numbers and properties can provide valuable information on therapy response and disease progression for patients with colorectal cancer. As several techniques for the detection of circulating tumor cells are notorious for yielding low detection rates in patients with non-metastatic colorectal cancer, the present study aimed to perform a proof-of-principle study using the Maintrac[®] approach for an assessment of circulating epithelial tumor cells (CETCs) in patients with colorectal cancer receiving neoadjuvant and/or adjuvant radio/chemotherapy (R/CT). CETCs in the peripheral blood of 22 patients with colorectal cancer were quantified by fluorescence image analysis (Maintrac[®]) before and after the first cycle of a neoadjuvant and/or adjuvant R/CT, as well as before and after surgical resection of the primary tumor. To determine that blood-borne CETCs originate from tumor tissues, spheres were cultured from CETCs as well as from primary tumor tissue and compared with the expression of tumor-specific antigens. Within the scope of this study, it was demonstrated that the Maintrac[®] method allows for the precise detection and characterization of CETCs in the blood of patients with colorectal cancer independent of tumor stage. Furthermore, correlations between CETC parameters and patients' response to neoadjuvant and/or adjuvant R/CT that

have been described in previous literature could be reproduced. Whether the observed trends are of a general nature and suitable as an auxiliary criterion for prognosis and treatment decisions remains to be shown. Patients with rectal cancer may benefit from CETC monitoring as a method to select suitable patients for adjuvant therapy.

Introduction

For both sexes, colorectal cancer is the second leading cause of cancer-related death, globally (9.2%) (1). The growing incidence, especially in industrialized countries, can be attributed to a change in lifestyle connected with obesity, physical inactivity, alcohol consumption and high red meat intake (2). Colorectal cancer is the result of a stepwise transition from normal mucosa to an invasive tumor, comprising several intermediate stages of premalignant or invasive lesions. As this process often drags on for years, cancer prevention and early diagnosis through screening programs represent a mainstay in colon cancer assessment and avoidance (3). Symptoms are generally associated with large tumors or advanced disease stages, and in most cases are relatively unspecific, so that the majority of colorectal cancers go unnoticed in early stages (3). Therapeutic options for the treatment of malignant tumors are resection, radiation and/or chemotherapy, depending on tumor stage and patient characteristics (3-5).

In the last 30 years, the survival of patients suffering from colorectal cancer has increased markedly, owing mainly to the introduction of screening programs and of new therapeutic agents (6). Conventional cytotoxic chemotherapy is the backbone of treatment for colorectal cancer patients with lymph node positive disease (7). Over the last decade, targeted therapies came to the fore with genomic markers enabling the selection of appropriate patients, who generally represent a minority among the whole patient population (8). But with the latest results regarding total neoadjuvant therapy (TNT), also cytotoxic chemotherapy gains in importance again. Both, the RAPIDO-, as well as the PRODIGE 23-study showed a significant and clinically relevant extension of disease-free survival after TNT instead of conventional, neoadjuvant RCT (9,10). Although some prognostic indicators

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Abbreviations: CETCs, circulating epithelial tumor cells; EGF, epidermal growth factor; EpCAM, epithelial cell adhesion molecule; FBS, fetal bovine serum; PD-L1, programmed death-ligand 1

Key words: circulating tumor cells, circulating epithelial tumor cells, Maintrac[®], colorectal cancer

for the probable response to conventional chemotherapy were identified, most of the proposed biomarkers and predictive assays are not currently used in the clinic, because of lacking validation, practicability and scalability, and of long turnaround times, or extensive costs (8,11-13). Altogether, there is a great demand for analytical methods easy-to-apply, which may support physicians with therapy decisions, and help to protect patients from under- or over-treatment.

Circulating tumor cells, readily accessible from blood samples of patients with solid tumors, are an important link between primary tumors and metastases. A longitudinal monitoring of their numbers and properties can provide valuable information on therapy response and disease progression. Various studies demonstrated a correlation between circulating tumor cells and metastases, survival and therapy response for patients with different types of cancer (14-18).

Ki-67 is a non-histone nuclear protein, which is expressed in actively proliferating cells throughout the cell cycle, but not in quiescent (G0) cells (19). Besides its detection in primary tumors, Ki-67 was also shown to be expressed in circulating tumor cells (20), and so might constitute a biomarker for identifying patients at a high risk of metastatic relapse.

While circulating tumor cells were shown to have prognostic potential for tumors of different entities (14-18), their clinical importance in colorectal cancer remained unclear. Our study was designed to use the immunofluorescence-based Maintrac® method to identify and quantify circulating epithelial tumor cells (CETCs) in the blood of patients with colorectal carcinoma (ICD10: C18/20) before and during neoadjuvant and/or adjuvant R/CT. Moreover, the ratio of CETCs expressing the proliferation marker Ki-67 was determined during the course of therapy.

Materials and methods

Patient and inclusion criteria. A total of 22 patients, diagnosed with colorectal cancer, were enrolled in this study between October 2018 and August 2020. Before treatment, all patients passed a complete clinical evaluation including clinical history, physical examination, rectoscopy/colonoscopy, relevant blood examination and chest/abdominal computed tomography. Local stage was determined according to the TNM classification of the UICC (21). The recruitment criteria were as follows: Histologically confirmed, invasive colorectal carcinoma (ICD10: C18/C20); primary diagnosis. The characteristics of all patients enrolled in this study are shown in Table I. All patients were treated according to current treatment guidelines for colon (ICD10: C18) or rectal (ICD10: C20) cancer (22). Long term R/CT for rectal cancer was performed as follows: Radiation dose: 50.4 Gy (single dose 1.8 Gy); target volume: Rectal cancer and region of pelvic lymphatic drainage; chemotherapy: 5-Fluorouracil (10 patients), Capecitabine (1 patient), 5-Fluorouracil/Oxaliplatin (3 patients). Individual therapy decisions were within the discretion of the attending physician and independent of any data collected in the course of this study. For patients receiving neoadjuvant therapy, 7.5 ml peripheral blood samples were obtained 1-7 days before initiation of R/CT, 17±3 days after the first cycle of R/CT, and after the completion of R/CT (1-7 days before surgery).

For patients with only or additional adjuvant therapy, blood samples were obtained 1-7 days before surgery, 6-8 weeks after surgery (before initiation of adjuvant therapy), 17±3 days after the first cycle of adjuvant chemotherapy, and on the last day of therapy, respectively.

The study was based on the Ethics Declaration of Helsinki and was approved by the Ethics Committee of the University of Bayreuth. Participants provided their written informed consent to participate in this study.

Assessment of tumor regression after neoadjuvant therapy. For all rectal cancer patients, treatment responses were assessed according to the pathological results after surgery, and graded by histological evaluation of the surgical specimens according to the criteria described by Dworak *et al.* (23). The grade of tumor regression was defined as follows: Grade 0: No regression; Grade 1: Dominant tumor mass with obvious fibrosis and/or vasculopathy; Grade 2: Dominantly fibrotic changes with few tumor cells or groups (easy to find); Grade 3: Very few tumor cells (difficult to find microscopically) in fibrotic tissue with or without mucous substance; Grade 4: No tumor cells, only fibrotic mass (total regression/response).

For a proper assessment of therapy response, we additionally compared the tumor size and lymph node status as assessed by computed tomography and/or endosonography of each patient before and after neoadjuvant R/CT. According to Dworak regression grade, as well as TNM re-staging, each patient was individually assigned either to the group of good or poor responders to neoadjuvant R/CT (Table II).

Blood collection and Maintrac® analysis. Peripheral blood (7.5 ml) from 22 patients with colorectal cancer at different stages of disease was drawn into blood count tubes containing ethylenediaminetetraacetic acid (EDTA) as an anticoagulant and processed 24 h after collection.

The Maintrac® approach was used for identification, quantification and further characterization of CETCs (22). To this end, 1 ml of EDTA-blood was subjected to red blood cells lysis at 4°C for 15 min using 14 ml erythrocyte lysis buffer (Qiagen GmbH). Remaining cells were spun down at 700 x g for 7 min at rt and resuspended in 500 µl of PBS/EDTA buffer. Immunostaining was performed by adding 4 µl fluorescein-isothiocyanate (FITC)-conjugated anti-human epithelial cell adhesion molecule (EpCAM) antibody (clone HEA-125; Miltenyi Biotec GmbH) to 25 µl of the cell suspension (about 10⁷ cells/100 µl) and incubation for 20 min at 4°C in the dark. The corresponding isotype control for EpCAM (mouse IgG1 K FITC; Miltenyi Biotec) was used at the same final concentration. In case of co-staining of Ki-67, additional 2.5 µl of phycoerythrin (PE)-conjugated anti-Ki-67 antibody (clone B56; BD Biosciences) was added prior to incubation. Subsequently, all samples were diluted in PBS/EDTA buffer to a total volume of 250 µl. A defined volume of the cell suspension and propidium iodide (PI; Sigma-Aldrich; Merck KGaA) was transferred to the wells of ELISA-plates (Greiner Bio-one). Co-staining of cells with Ki-67 was performed without PI. Red and green fluorescence of the cells was examined using a Fluorescence Scanning Microscope ScanR (Olympus), enabling detection and relocation of cells for visual examination of EpCAM-, PI- or Ki-67-positive cells.

Table I. Clinicopathological characteristics of patients with colon (C18) and rectal (C20) cancer included in this study.

Clinicopathological characteristics	Number of patients with colon cancer, n (%)	Number of patients with rectal cancer, n (%)
Total	6 (27)	16 (73)
Age, years		
>60	5 (23)	11 (50)
≤60	1 (4)	5 (23)
Sex		
Female	3 (14)	6 (27)
Male	3 (14)	10 (45)
Tumor size ^a		
T1	0 (0)	0 (0)
T2	1 (4)	3 (14)
T3	4 (18)	11 (50)
T4	1 (4)	2 (9)
Lymph node status ^a		
Positive	6 (27)	4 (18)
Negative	0 (0)	12 (54)
Distant metastasis		
Positive	1 (4)	1 (4)
Negative	5 (23)	15 (68)
Neoadjuvant therapy	1 (17) ^b	14 (64)
Adjuvant therapy	6 (27)	3 (14)

^aObtained by histopathological examination of a surgical specimen;

^bone patient with carcinoma of the colon ascendens also had rectal cancer, for which neoadjuvant radiochemotherapy had been performed prior to study entry.

For quantification of CETCs, only vital CETCs with intact cell morphology and without PI staining were counted. For daily verification of optical components and detectors of the microscope, fluorospheres (Flow-Check 770; Beckman Coulter) were used.

Culture of spheres from peripheral blood. Only a small subpopulation of CETCs possessing additional stem cell properties is able to grow into metastases. By enumeration of CETCs able to clonally grow into CETC microspheres under specific conditions, we specified and quantified this subpopulation. Therefore, CETCs and leukocytes were isolated from peripheral blood as described earlier, plated at a density of 2×10^5 cells/ml in RPMI-1640 supplemented with l-glutamine, HEPES, penicillin/streptomycin and growth factors such as EGF, insulin and hydrocortisone, and incubated under standard cell culture conditions (37°C, 5% CO₂) in a sterile incubator. Every five days, the cultures were inspected under an inverted light microscope (PrimoVert) and fresh culture medium was added. Between days 21 and 28 of incubation, spheres were collected from the culture flasks, pelleted (250 x g, 7 min), and resuspended in 500 μl PBS. Immunostaining of spheres

was performed using FITC-conjugated mouse anti-human EpCAM-antibody (clone HEA-125; Miltenyi Biotec GmbH), PE-conjugated mouse anti-human CD44-antibody (BD Biosciences) or mouse anti-human CD133-antibody (clone 7; BioLegend) for 20 min at 4°C in the dark. The samples were then diluted in PBS/EDTA and transferred into the wells of a 96-well microtiter plate (Greiner Bio-one). Analysis of fluorescence was performed using a fluorescence scanning microscope (ScanR; Olympus). To verify vitality, PI staining of spheres was performed before the analysis. Finally, only vital CTC spheres with intact morphology and without PI staining were counted.

Primary culture from tumor tissue. In case of surgery of the primary tumor, a small piece of tissue from the middle of the tumor (ø depending on the size of the tumor) was obtained in a sterile falcon in 10 ml transportation medium (RPMI-1640, 5% FBS, 5 μg/ml insulin, 2.75 μg/ml transferrin, 20 mM sodium selenite, 55 μg/ml sodium pyruvate, 1 μM hydrocortisone, 1,000 U/ml penicillin, 1,000 μg/ml streptomycin, 250 mg/ml amphotericin B, 15 mM HEPES, 100 μg/ml gentamycin, 5 μg/ml metronidazole) directly from the operating theater and kept at 4°C for transportation. All samples were processed within 24 h after withdrawal. For further processing, the tumor tissue was washed 3-5 times in PBS by extensive shaking and put into a sterile petri dish. Before the tissue was chopped into small pieces of about 1 mm in diameter by anti-parallel movement of two scalpels, it was covered with a small amount of sphere culture medium (RPMI-1640 supplemented with l-glutamine, HEPES, penicillin/streptomycin and growth factors such as EGF, insulin and hydrocortisone). After one more washing step with PBS, the tissue was enzymatically homogenized with Accumax™ solution (Sigma-Aldrich; Merck KGaA) for 45 min under continuous mixing at rt. Then the cell suspension was filtered using a cell strainer (mesh size 0.44 μm; Greiner Bio-one) to eliminate bigger cell clumps and centrifuged at 240 x g for 10 min at rt. The resulting cell pellet was resuspended in 1 ml of culture medium and the number of vital cells was determined by bromophenol blue staining. Finally, the cells were plated in a concentration of approximately 0.6×10^6 vital cells/ml in culture medium in 6-well plates and incubated at standard cell culture conditions (37°C, 5% CO₂) for several weeks. All cultures were checked for bacterial infections daily and in the case of a minor infection isolated and treated with additional antibiotics, or in case of a major infection, discarded. If primary tumor spheres were detectable after a few weeks, a small amount of the culture was harvested and immunostained for further characterization and documentation.

Statistical analysis. Statistical analysis was performed using SigmaPlot (version 14.0; Systat Software Inc.) for Windows. Comparisons between variables were performed using ANOVA (analysis of variance) followed by a post hoc test for parametric data, or Kruskal-Wallis test followed by Dunn's test for nonparametric data. The significance level was set at P<0.05.

Results

General. A total of 22 patients with histologically confirmed colorectal cancer (16 patients with rectal cancer, 6 patients

Table II. Responses of patients with rectal cancer (C20) after receiving neoadjuvant R/CT.

Patient number	TNM		Regression grade	Response category
	Before R/CT	After R/CT		
1	μ , T2; μ , N0	yp, T3b; yp, N1	1	Poor
2	μ , T2; c, T3; μ , N1	yp, T2; yp, N0	3	Good
3	μ , T3; μ , N+	yp, T2; yp, N0	2	Good
4	μ , T3; μ , N0	yp, T2; yp, N0	3	Good
5	μ , T3; μ , N1; c, M1HEP	yp, T3; yp, N0	3	Good
6	c, T4; c, N2b	yp, T3b; yp, N0	3	Good
7	c, T3; c, N+	yp, T3a; yp, N1b	3	Good
8	μ , T3; μ , N+	yp, T3a; yp, N0	3	Good
9	μ , T3; μ , N0	yp, T3b; yp, N0	1	Poor
10	μ , T2; μ , N+	yp, T3; yp, N0	2	Poor
11	c, T3; c, N1	yp, T4a; yp, N1b	3	Poor
12	μ , T2; μ , N+	yp, T3a; p, N0	1	Poor
13	μ , T3; μ , N1	yp, T3b; yp, N0	1	Poor
14	μ , T3; μ , N1; c, M1aPUL	yp, T4a; yp, N0; c, M1a	1	Poor

Patients with rectal cancer (C20) were assigned to either the group of good or poor responders according to Dworak regression grade and TNM re-staging. μ , stage determined by ultrasonography; c, stage determined by clinical examination; y, stage assessed after R/CT; p, stage given by histopathological examination of a surgical specimen; TNM, tumor node metastasis; R/CT, radio/chemotherapy.

with colon cancer) were enrolled in this study. Patients' characteristics are given in Table I.

Considering all patients (ICD10: C18 and C20), 1 patient was at stage I (4%), 7 patients were at stage II (32%), 12 patients were at stage III (55%), and 2 patients were at stage IV (9%). Six patients (27%) suffered from colon cancer and 16 (73%) from rectal cancer. The age of the patients ranged from 51 to 80 years (median 65.5 years). The median number of CETCs of all 22 patients with colorectal cancer was 55 CETCs per 100 μ l cell suspension (ranging from 0 to 640) from which colon cancer patients (ICD10: C18) had a median CETC number per 100 μ l of 45 (ranging from 0 to 145), and rectal cancer patients (ICD10: C20) of 65 (range from 0 to 640). No statistically significant differences in CETC numbers were observed in correlation to tumor size, lymph node status or distant metastasis (data not shown).

CETC quantification. Using the Maintrac® method we detected CETCs in 100% of colorectal cancer patients included in this study. CETC numbers of all patients during the course of therapy are specified in Table SI. In addition to epithelial characteristics as assigned by immunostaining, we could demonstrate proliferative and stemness properties of CETCs under specific conditions, which were identical to those of cells derived from the primary tumor itself (Fig. 1).

CETC characterization. CETCs from patient #1 were investigated for their proliferative activity by growing non-adhesive suspension cultures. Formation of EpCAM-positive spheres was observed after the first cycle of neoadjuvant R/CT (5 spheres/100 μ l blood). Interestingly, CETCs from all other samples did not show any sphere formation. During surgery of patient 1, a small piece of

tumor tissue was set aside and stored on ice until further processing. After separation and washing, primary tumor cells were cultured under the same conditions as CETCs, also resulting in the formation of spherical structures. Both, spheres from the primary tumor, as well as spheres from the peripheral blood of patient #1 were further characterized by immunostaining (Fig. 1). The viability of the spheres was ensured by counterstaining with PI (propidium iodide), which cannot permeate live cells. Expression patterns in primary tumor spheres of specific stem cell markers, which are regularly over-expressed in colorectal tumors (CD44 and CD133), correlated with those in spheres from peripheral blood. Moreover, primary tumor spheres expressed high levels of PD-L1.

Response to neoadjuvant R/CT in rectal cancer patients.

14 patients with rectal cancer received neoadjuvant R/CT, 7 (50%) of whom showed a good response (Fig. 2) and 7 (50%) did not or only partially respond to the therapy (Fig. 3). In the group of good responders, the mean CETC number before R/CT was 105 CETCs/100 μ l of cell suspension. After the first cycle of the R/CT it decreased to 47 per 100 μ l. With a P-value of 0.543, the differences between the three time points did not reach statistical significance likely due to the small sample size. Nevertheless, the results show a trend. In detail, the CETC numbers declined in 5 of 7 patients (71%) and increased in only 2 patients (29%) with good response to neoadjuvant R/CT (Fig. 2). In the group of poor responders (7 patients), the mean CETC number was initially 40 CETCs/100 μ l cell suspension, and increased continuously from 73 after the first cycle of the R/CT to 210 CETCs/100 μ l cell suspension before surgery (Fig. 3). Again, the small number of participants (n=7) might be the major cause for the lack of statistical significance (P=0.428).

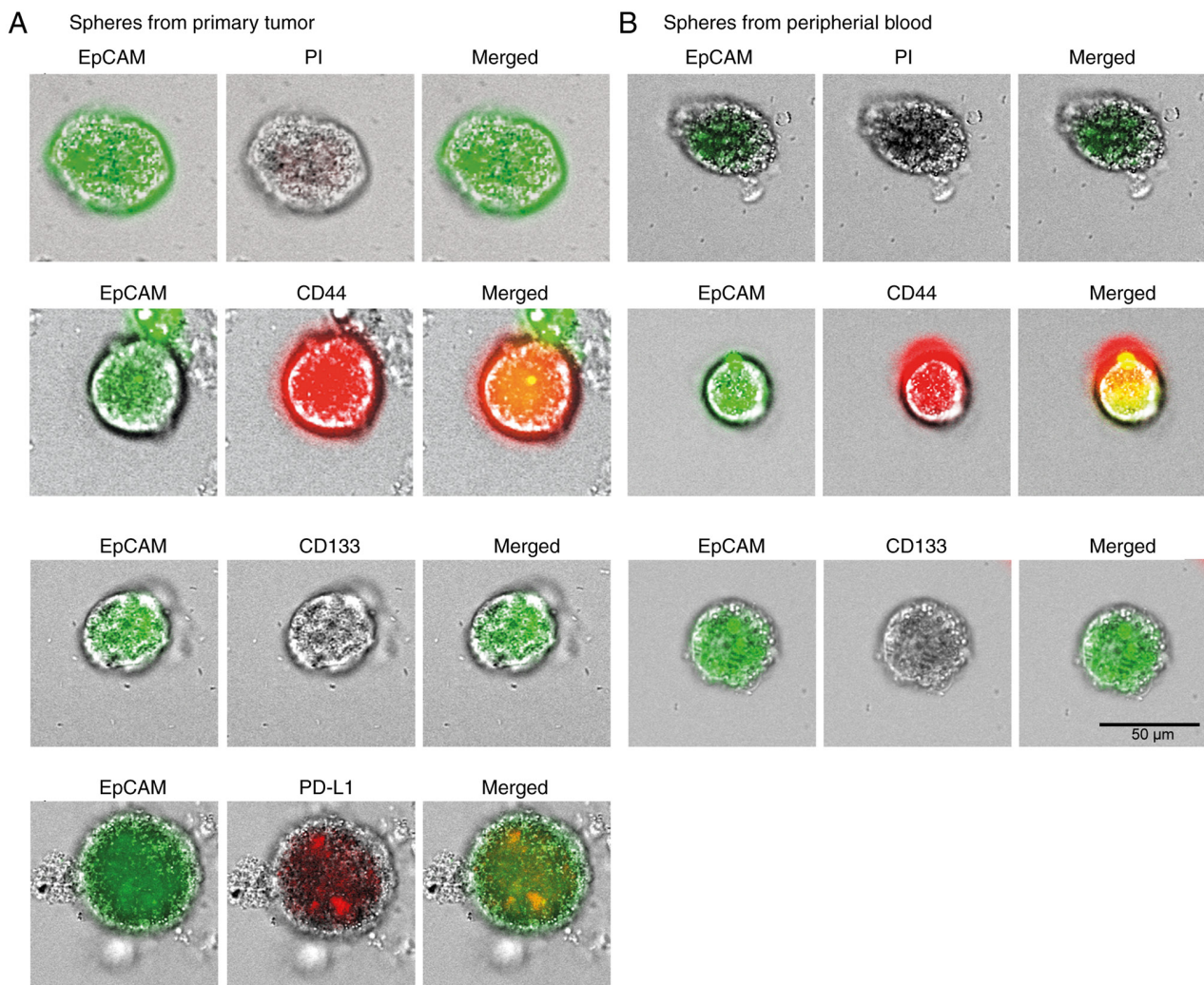


Figure 1. 3-Dimensional spheres cultured from peripheral blood or primary tumor tissue from patient #1. Immunostaining of spheres cultured from (A) primary tumor tissue or (B) CETCs from peripheral blood from patient #1. The epithelial origin of the spheres was identified by staining with anti-EpCAM antibody. PI was used as a vitality marker. Additionally, EpCAM-positive spheres were investigated for the expression of specific stem cell markers (CD44, CD133) and PD-L1. EpCAM, epithelial cell adhesion molecule; PI, propidium iodide; PD-L1, programmed death-ligand 1; CETC, circulating epithelial tumor cell.

Response to adjuvant therapy in colorectal cancer patients. 9 patients (41%; 6 patients with colon cancer and 3 patients with rectal cancer) received adjuvant chemotherapy and CETCs were quantified before surgery, before the beginning of chemotherapy and after the first cycle of adjuvant therapy (Fig. 4). Before surgery the median CETC number was 55/100 µl cell suspension, 6-8 weeks after surgery (before the beginning of the adjuvant CT) the median value was 65, and after the first cycle of CT the median was 20 CETCs/100 µl cell suspension. The difference between the mean values of the three time points was not statistically significant ($P=0.114$).

Interestingly, all patients showed decreasing CETC numbers under adjuvant chemotherapy.

Expression of the proliferation marker Ki-67 during therapy. Ki-67-positive CETCs were detected in 20 patients (91%) and the percentage ranged from 0-100 (median: 25 Ki-67-positive CETCs/100 µl cell suspension). The median of Ki-67-positive CETCs/100 µl cell suspension in colon cancer patients was 18 (ranging from 0 to 170), and in rectal cancer patients 25 (ranging from 0 to 169). Although the differences in the

Ki-67-positive CETCs at the three time points were not statistically significant neither for patients with neoadjuvant R/CT ($P=0.202$), nor in the group of patients with adjuvant CT ($P=0.151$), there was a trend in the number of Ki-67-positive CETCs to decrease under adjuvant CT, and to increase in patients receiving neoadjuvant R/CT (Fig. 5).

Case report. Fig. 6 shows an example of a serial analysis of the CETC numbers during the therapy of a 63-year-old patient with stage III (T3, N1, M0; G2) rectal cancer. The patient was treated with neoadjuvant R/CT (Dworak 1, poor response), followed by surgery (R0-resection) and additional adjuvant chemotherapy. During neoadjuvant therapy the CETC numbers increased significantly and reached their maximum (225 CETCs/100 µl cell suspension) before surgical removal of the primary tumor. Eight weeks after surgery the CETC number had fallen to a level similar to that at the beginning of the R/CT. It continued to decrease until there were no residual CETCs detectable at the last day of the adjuvant CT. Until 9 months after completion of the adjuvant therapy, this patient has remained free of relapse.

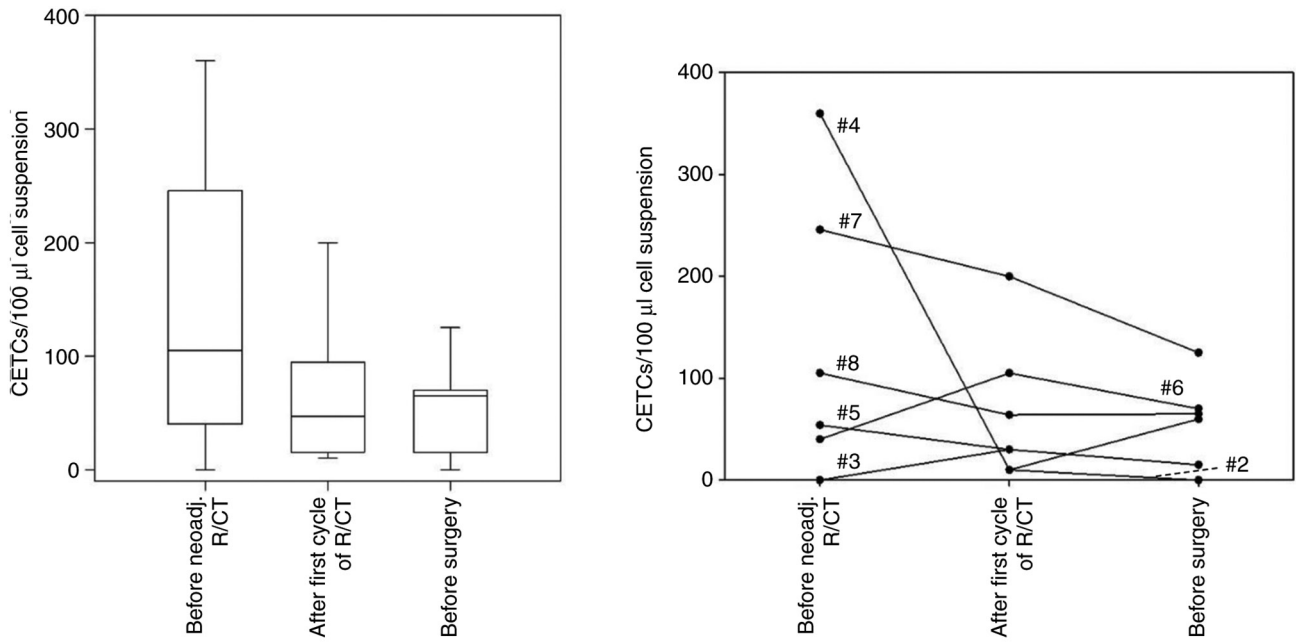


Figure 2. Number of CETCs in the blood of patients with rectal cancer with good response to neoadj. R/CT. Blood samples were drawn before R/CT, after the first cycle of R/CT and after completion of R/CT immediately before surgery. Left, boxplot with median CETC values, quartiles and variability at each time point; right, individual CETC numbers at all time points, each line represents one patient. Patient #4 (360/10/60 CETC/100 μ l), patient #7 (246/200/125 CETC/100 μ l), patient #8 (105/64/65 CETC/100 μ l), patient #5 (54/30/n.d. CETC/100 μ l), patient #6 (40/105/70 CETC/100 μ l), patient #3 (0/30/15 CETC/100 μ l), patient #2 (n.d./10/0 CETC/100 μ l). Assignment of patients in Tables II and SI. n.d., not defined; CETC, circulating epithelial tumor cell; neoadj., neoadjuvant; R/CT, radio/chemotherapy.

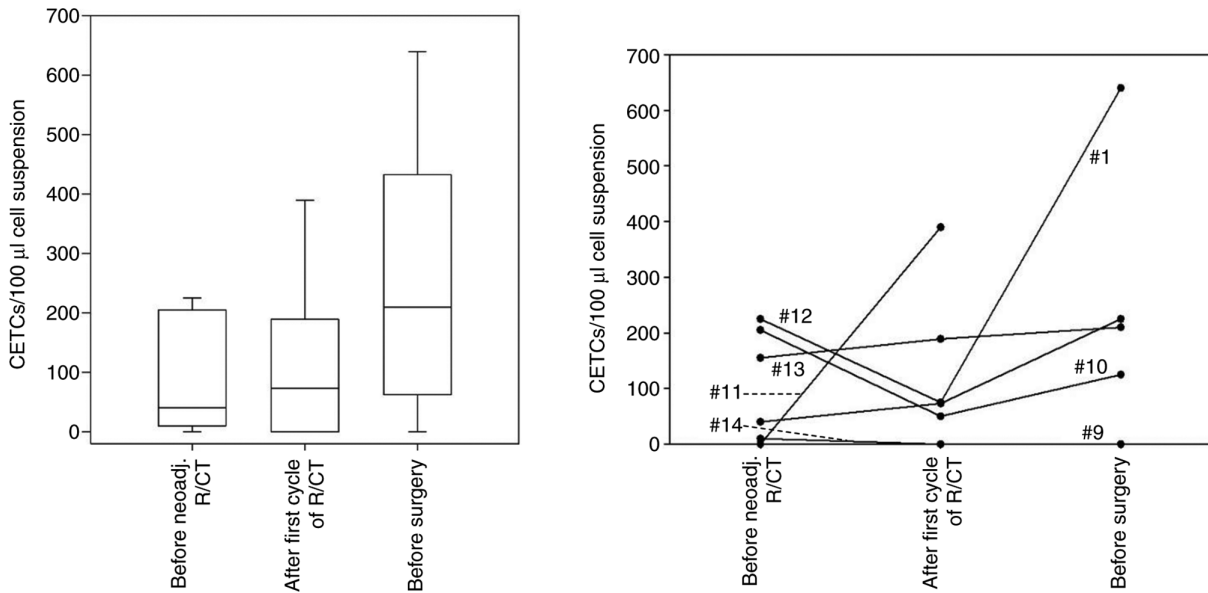


Figure 3. Number of CETCs in the blood of patients with rectal cancer with poor response to neoadj. R/CT. Blood samples were drawn before R/CT, after the first cycle of R/CT and after completion of R/CT immediately before surgery. Left, boxplot with median CETC values, quartiles and variability at each time point; right, individual CETC numbers at all time points, each line represents one patient. Patient #1 (40/73/225 CETC/100 μ l), patient #9 (10/0/0 CETC/100 μ l), patient #10 (205/50/125 CETC/100 μ l), patient #11 (0/390/n.d. CETC/100 μ l), patient #12 (225/75/640 CETC/100 μ l), patient #13 (155/189/210 CETC/100 μ l), patient #14 (10/0/n.d. CETC/100 μ l). Assignment of patients in Tables II and SI. n.d., not defined; CETC, circulating epithelial tumor cell; neoadj., neoadjuvant; R/CT, radio/chemotherapy.

Discussion

Although disseminated tumor cells play a major role in the metastatic process of tumors, their detection and monitoring does not play a decisive role in standard clinical procedures. Monitoring of circulating tumor cells in the blood of cancer

patients during therapy has already been shown to be a powerful prognostic tool for tumors of different entities including colorectal tumors (15,24-26). From a clinical perspective, assessment of patients' response to antitumoral therapy by detection of circulating tumor cells in the peripheral blood appears comfortable, both for the physician (time- and

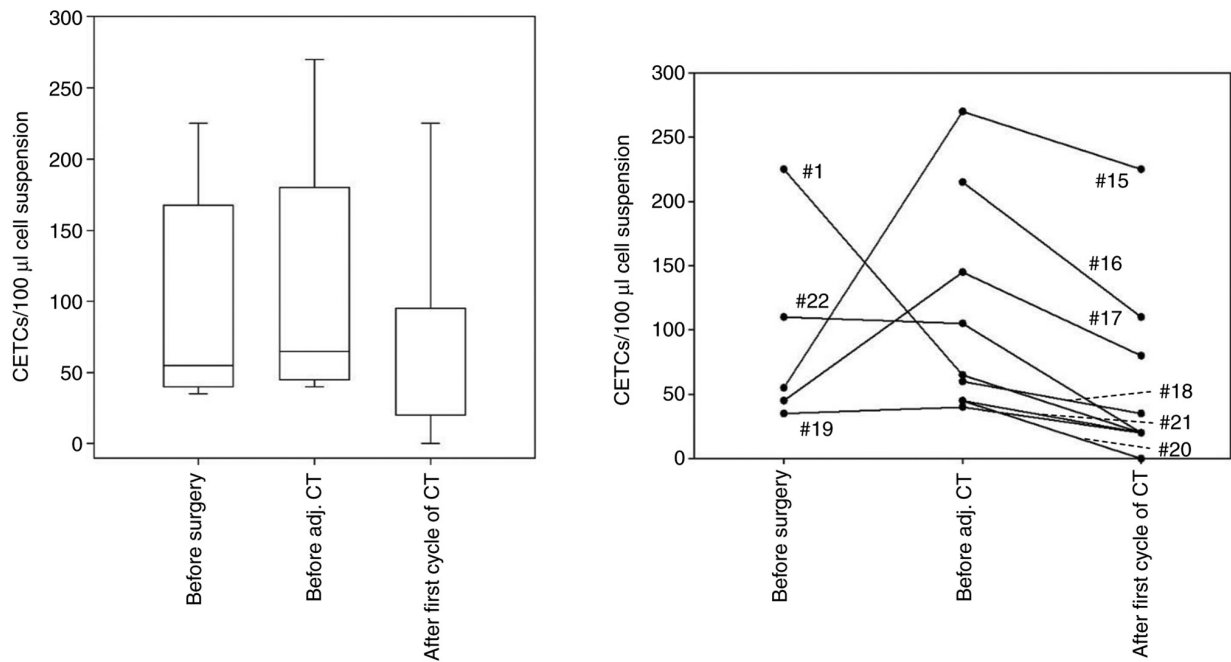


Figure 4. Number of CETCs in the blood of patients with colorectal cancer with adj. CT. Blood samples were drawn directly before surgery, 6-8 weeks after surgery and after the first cycle of adj. CT. Left, boxplot with median CETC values, quartiles and variability at each time point; right, individual CETC numbers at all time points, each line represents one patient. Patient #1 (C20; 225/65/20 CETC/100 µl), patient #15 (C20; 55/270/225 CETC/100 µl), patient #16 (C20; n.d./215/110 CETC/100 µl), patient #17 (C18; 45/145/80 CETC/100 µl), patient #18 (C18; n.d./60/35 CETC/100 µl), patient #19 (C18; 35/40/20 CETC/100 µl), patient #20 (C18; n.d./45/0 CETC/100 µl), patient #21 (C18; n.d./45/20 CETC/100 µl), patient #22 (C18; 110/105/20 CETC/100 µl). Assignment of patients in Table SI. n.d., not defined; CETC, circulating epithelial tumor cell; adj., adjuvant; CT, chemotherapy; C18, colon carcinoma; C20, rectum carcinoma.

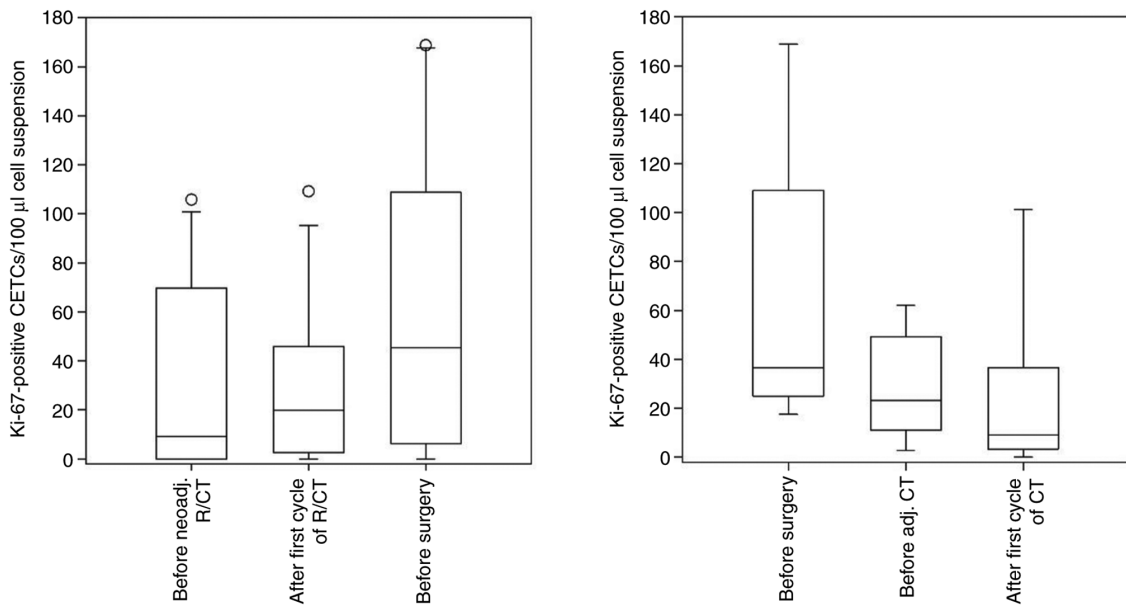


Figure 5. Boxplots with median values, quartiles and variabilities of Ki-67-positive CETCs in the blood of patients with colorectal cancer. Left, patients with neoadj. R/CT; blood samples were drawn before the beginning of the neoadj. R/CT, after the first cycle of R/CT and after completion of R/CT (before surgery). Right, patients with adj. CT; blood samples were drawn directly before surgery, 6-8 weeks after surgery and after the first cycle of CT. CETC, circulating epithelial tumor cell; neoadj., neoadjuvant; adj., adjuvant; CT, chemotherapy; R/CT, radio/chemotherapy.

cost-efficient), as well as for the patients (non-invasive, neither toxic nor painful), and may be easily repeated as a monitoring tool without great efforts.

In recent years, different techniques have been described for the detection of circulating tumor cells (27-32). Various studies demonstrated that their detection via CellSearch system could

be used to predict treatment responses and long-term prognosis for stage IV colorectal cancer patients (33,34). But in the case of non-metastatic patients, the detection rate via CellSearch system is too low (11-25%) to further analyze the correlation between circulating tumor cells and patients' characteristics and treatment responses (35). In the present *proof-of-principle*

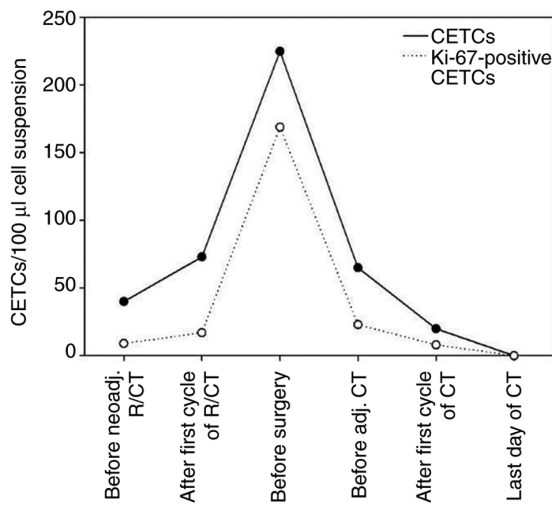


Figure 6. Number of CETCs and Ki-67-positive CETCs in the blood of patient #1 with rectal cancer receiving neoadj. R/CT, as well as adj. CT after surgical removal of the primary tumor (R0-resection). Blood samples were drawn prior to the neoadj. R/CT (40 CETCs/100 µl), after the first cycle of R/CT (73 CETCs/100 µl), immediately before surgery (225 CETCs/100 µl), 6-8 weeks after surgery/before the beginning of adj. CT (65 CETCs/100 µl), after the first cycle of adj. CT (20 CETCs/100 µl) and on the last day of adj. CT (0 CETCs/100 µl). CETC, circulating epithelial tumor cell; neoadj., neoadjuvant; adj., adjuvant; CT, chemotherapy; R/CT, radio/chemotherapy.

study with a small group of colorectal cancer patients of all stages using the Maintrac® method for CETC identification, we detected CETCs in the peripheral blood of 100% of the patients. Moreover, we were able to show that some of the cells, which have been quantified, possess proliferative and stemness characteristics matching those found in the primary tumor.

Up until now, only a few studies investigated the role of circulating tumor cells for evaluating the response to neoadjuvant R/CT for patients with rectal cancer. In a study by Zitt *et al* the circulating tumor cells were investigated during neoadjuvant R/CT in 26 patients with locally advanced rectal cancer using a non-quantitative RT-PCR-based method (36). Sun *et al* used a size-dependent detection method to analyze changes in circulating tumor cell numbers during neoadjuvant R/CT within a collective of 115 rectal cancer patients (37). Keeping in mind the respective drawbacks of each method, both studies agreed, that responders had an obvious decrease of the numbers of circulating tumor cells after neoadjuvant R/CT, while there was no noticeable alteration after treatment in non-responders. These results were confirmed by other studies (38,39). In our study, applying the Maintrac® approach for CETC detection, we allocated 14 rectal cancer patients either to the group of good or poor responders to neoadjuvant R/CT, based upon alterations in TNM staging before and after R/CT and on Dworak regression grades of their surgical specimens. For both groups we confirmed that a decrease in CETC numbers correlates with, and thus indicates, a good response, whereas an increase of CETC numbers is rather indicative of a poor response to neoadjuvant R/CT using the Maintrac® approach for CETC detection. Whether this observation is of general relevance, and thus a potential prognostic tool for the clinician, must be clarified by extended studies with larger collectives of

patients. As all patients in our study experienced a decline of CETCs under adjuvant treatment, it would be interesting to know if and when individual rectal cancer patients benefit from an adjuvant chemotherapy after neoadjuvant R/CT. Moreover, it would also be interesting to see, if CETC monitoring may also be able to identify individual rectal cancer patients, which benefit from TNT. The latest results of the PRODIGE 23 and RAPIDO phase III clinical studies have shown that TNT is able to extent disease free survival, as well as to improve the pathological complete remission (pCR) rate, organ preservation and local control in patients with locally advanced rectal cancer in comparison to conventional, neoadjuvant/adjuvant therapy regimes (9,10).

As discussed above, we observed a heterogenic reaction of the CETC profile for patients receiving neoadjuvant R/CT. In contrast, a constant decrease in CETC numbers during adjuvant therapy was found. A potential explanation for this discrepancy may be the tumor burden in the adjuvant versus the neoadjuvant situation. While the neoadjuvant therapy targets the whole, intact tumor, in the adjuvant situation the tumor burden is low, because only microscopic tumor residues remain in the patient after surgery which may be more sensitive to chemotherapy and radiation. In addition, because of the reduced number of tumor cells, the development of resistance is less likely when compared to the neoadjuvant situation (40,41).

The proliferation marker Ki-67 is widely utilized in routine clinical diagnostic of breast cancer patients (42). Lumachi *et al* suggested Ki-67 as a predictive parameter for colorectal cancer, as they found an inverse correlation between Ki-67 expression and overall survival in a small retrospective study (43). However, its prognostic value for colorectal tumors remains controversial. While some studies completely failed to demonstrate its prognostic significance in the case of colorectal tumors (44), others found Ki-67 overexpression indicating a good clinical outcome for colorectal cancer patients (45). In our study, we observed that the Ki-67 index, and thus the proliferative activity, of CETCs from the blood of colorectal cancer patients increased during neoadjuvant R/CT, and decreased during adjuvant CT. Considering the above mentioned findings from literature, these results cannot be interpreted regarding a good or poor prognosis for the patients. On a cellular level, one possible explanation for the rise in Ki-67 expression under neoadjuvant R/CT may be the radiotherapy-induced inflammation, which in turn induces an increase of the proliferating activity of tumor cells, and consequently of circulating tumor cells (46,47).

Finally, the general trends reported in this study could be exemplified by a case report of a rectal cancer patient (#1) receiving neoadjuvant R/CT, as well as adjuvant CT. This patient seemed to benefit from the surgery and from the additional adjuvant CT as CETC numbers decreased continuously after surgery to reach zero level on the last day of adjuvant therapy. This patient has remained free of relapse until nine months after the completion of therapy.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

RS, AK, KP and MG contributed to the design of the present study and developed the methodology. MG collected the bioinformatics data, performed the experiments, analyzed the results and wrote the manuscript. AK contributed to the collection of patient data. RS, AK and KP critically revised the manuscript and approved the final version to be published. All authors agreed to be accountable for all aspects of the study. All authors have read and approved the final manuscript. MG and RS confirm the authenticity of all the raw data.

Ethics approval and consent to participate

The present study was approved by the Ethics Committee of the University of Bayreuth (approval no. O 1305/1-GB; Bayreuth, Germany). Written informed consent was obtained from all patients.

Patient consent for publication

Not applicable.

Competing interests

Katharina Pachmann holds a patent protecting the Maintrac® method used in the present study (patent no. EP 3128325 B1; dated February 8th, 2017). The other authors declare that they have no competing interests.

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