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
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 macroscopically) 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 10³ 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 phycocerythrin (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.
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 before the analysis. Finally, only vital CTC spheres with intact morphology and without PI staining were counted.

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<sup>a</sup>            |                                             |                                             |
| T1                                | 0 (0)                                       | 0 (0)                                       |
| T2                                | 1 (4)                                       | 3 (14)                                      |
| T3                                | 4 (18)                                      | 11 (50)                                     |
| T4                                | 1 (4)                                       | 2 (9)                                       |
| Lymph node status<sup>a</sup>     |                                             |                                             |
| 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)<sup>b</sup>                         | 14 (64)                                     |
| Adjuvant therapy                  | 6 (27)                                      | 3 (14)                                      |

<sup>a</sup>Obtained by histopathological examination of a surgical specimen; <sup>b</sup>one 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 2x10<sup>3</sup> 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% CO<sub>2</sub>) 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 scalpsels, 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<sup>®</sup> 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 0.6x10<sup>6</sup> vital cells/ml in culture medium in 6-well plates and incubated at 37°C, 5% CO<sub>2</sub> 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 non-parametric 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...
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 µl 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 µl of cell suspension. After the first cycle of the R/CT it decreased to 47 per 100 µl. 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 µl cell suspension, and increased continuously from 73 after the first cycle of the R/CT to 210 CETCs/100 µl 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).

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

| Patient number | Before R/CT | After R/CT | Regression grade | Response category |
|---------------|------------|------------|------------------|------------------|
| 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 ultrasoundography; 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.
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.
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
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...
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 extend 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.

Acknowledgements

Not applicable.
Funding

The present study was supported by a PhD fellowship from the Bayerische Eliteförderungsgesetz (BayEFG).

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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