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Circulating tumour cells as a predictive factor for response to systemic chemotherapy in patients with advanced colorectal cancer

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ABSTRACT

Circulating tumour cells (CTC) can be traced in patients with different types of cancer. The aim of this study was to detect CTC in patients with advanced colorectal cancer and whether CTC are still detectable after systemic chemotherapy. Blood from 34 patients with advanced colorectal cancer was analysed for the presence of CTC before chemotherapy was given and after 3 months. Eleven patients demonstrated a tumour remission after chemotherapy. In 6 cases CTC were detectable before but not after initiation of chemotherapy. Ten patients demonstrated a progression. In 5 cases CTC were detected before and after chemotherapy. Our data suggest that the detection of CTC will help to identify patients responding to chemotherapy or with a risk of a therapy failure.

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1. Introduction

The presence of circulating tumour cells (CTC) in the peripheral blood of patients with colorectal cancer (CRC) has already been noted (Cohen et al., 2006; Molnar et al., 2003; Zieglschmid et al., 2007) and their clinical relevance has been described extensively as an independent prognostic marker for disease-free survival (Guller et al., 2002; Koch et al., 2006; Thorban et al., 2006). CTC could be used as surrogate markers to monitor drug effects and clinical status (Elshimali and Grody, 2006). If a patient responds to an administered therapy, CTC should be no longer detectable in peripheral blood. Therefore, patients with CTC might be potential candidates for relapse and therapy failure.

Besides chemotherapeutic agents like 5-fluorouracil (5-FU), folinic acid (FA), oxaliplatin and irinotecan, targeted therapies including therapeutic antibodies are administered in combination or in chemotherapeutic resistant patients. Bevacizumab, panitumumab and cetuximab are already approved and the latter is used in daily practice with metastatic CRC patients as a target antibody for epidermal growth factor receptor (EGFR) (Pfeiffer et al., 2007). EGFR, a transmembrane receptor tyrosine kinase is activated by binding of the natural ligand epidermal growth factor (EGF) or transforming growth factor α (TGF α). The therapeutic antibodies cetuximab and panitumumab bind to EGFR with high affinity and therefore prevent the binding of EGF or TGF α . This results in reduced

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receptor tyrosine kinase activity and in reduced cell proliferation, cell survival and cell invasion.

In most studies patients with metastasised CRC have an extended time to tumour progression if they were treated with antibody based therapies independent of the EGFR status of the primary tumour (Meropol, 2005; Pfeiffer et al., 2007; Zhang et al., 2006). EGFR expression analysis by immunohistochemical staining of tumour sections correlates only in some cases with tumour response to EGFR targeted therapy and is therefore of limited help for the identification of patients possibly responding to EGFR antibody therapy (Chung et al., 2005; Cunningham et al., 2004; Nygren et al., 2005; Vallböhmer et al., 2005). Genetic alterations of the extracellular domain of the EGF receptor or different EGFR expression pattern in the primary tumour and metastasis or CTC can both lead to treatment failure despite immunohistochemically detected EGFR expression in tumour biopsies (Bralet et al., 2005; Italiano et al., 2005; Scartozzi et al., 2004).

This pilot study was performed to determine the predictive value of CTC. Blood obtained from CRC patients was examined by immunomagnetic enrichment with subsequent RT-PCR techniques for the circulation of tumour cells before and after therapy. Peripheral blood from patients with chronic inflammatory bowel disease was used as a control group to demonstrate the specificity of CTC detection on patients with colorectal cancer. We hypothesized that successful chemotherapy should lead to a decreased number of CTC, which will then lead to a negative PCR result. PCR results were compared with clinical responses. Another aim of the study was to determine the expression of EGFR variants on CTC to identify additional patients who might not profit from EGFR antibody targeted therapies because of the deletion of extracellular binding sites.

2. Patients and methods

2.1. Patients and study design

A total number of 34 CRC patients (20 males, 14 females) with advanced disease were enrolled before and 3 months after chemotherapeutic treatment at the Department of Gastroenterology, Hepatology and Endocrinology of the Hannover Medical School, Germany. Peripheral blood from 64 patients with Crohn's disease (13 males, 19 females) or ulcerative colitis (19 males, 13 females) was also tested to evaluate the specificity of the test used. Informed consent was obtained from all patients prior to obtaining blood samples; the Ethics Committee of the Hannover Medical School approved the study protocol. The following parameters were recorded for all patients: age, sex, diagnosis, serum carcinoembryonic antigen (CEA) and the presence of CTC. Additionally the following parameters were recorded for the CRC patients: TNM classification of the primary tumour, status of disease clinically validated by computer tomography before and 3 months after treatment classified according to WHO criteria.

2.2. Tumour cell enrichment and multiplex RT-PCR

Peripheral blood (2×5 ml) from each patient was collected in EDTA tubes (Sarstedt AG & Co, Nümbrecht, Germany) and

processed within 4 h for the enrichment of CTC and subsequent expression analyses. For the detection of CTC the AdnaTest ColonCancerSelect and AdnaTest ColonCancerDetect (AdnaGen AG, Langenhagen, Germany) were employed according to the manufacturer's protocol. The combination of immunomagnetic tumour cell enrichment and the analysis of tumour-associated transcripts EGFR, CEA and GA733-2 (gastrointestinal tumour-associated antigen 733-2) by multiplex RT-PCR were previously described (Zieglschmid et al., 2005). Actin was amplified as an internal PCR control. PCR products were analysed with DNA 1000 assays using an Agilent 2100 Bioanalyzer (Analysis Software 2100 expert, version B.02.03.SI307, Agilent Technologies, Böblingen, Germany). A threshold of <0.1 ng/ μ l was defined as negative. The cDNA was also used for the detection of different extracellular EGFR variants.

2.3. PCR amplification of extracellular EGFR variants from CTC

The PCR for the detection of extracellular EGFR variants in CTC was previously described (Lankiewicz et al., *in press*). In brief, the PCR was performed under the following conditions: Two primer pairs (EGFR P1 5'-AAACTGCACCTCCATCAGTG-3'/EGFR P2 5'-ATTTCGTTGGACAGCCTTCAAG-3' and EGFR P3 5'-GTCCAGTATTGATCGGGAGAGC-3'/EGFR P4 5'-GAGCCGTGA TCTGTGACCAC-3') were designed to span exon 9 to exon 16 and exon 1 to exon 8, respectively; 1.0μ M of each primer and 25μ l of HotStarTaq Mix (Qiagen GmbH, Hilden, Germany) were used. The PCR program was set for 15 min at 95°C , followed by 45 cycles of 94°C for 30 s, 60°C (for P1/P2) or 63°C (for P3/P4) for 30 s and 72°C for 1 min (for P1/P2) or 2 min (for P3/P4), followed by a final step of 72°C for 5 min. The analyses of the PCR fragments were performed with DNA 1000 (for fragments detected with P1/P2) or DNA 7500 assays (for fragments detected with P3/P4) and an Agilent 2100 Bioanalyzer (Analysis Software 2100 expert, version B.02.03.SI307, Agilent Technologies, Böblingen, Germany) according to the manufacturer's instructions.

2.4. Determination of serum CEA

CEA levels were determined using standard assays (Modular Analytics <E 170>, Roche Diagnostics GmbH, Mannheim, Germany) according to the manufacturer's instructions. The results were evaluated using a threshold of >3 mg/l defined as positive.

3. Results and discussion

3.1. Detection of CTC and determination of CEA serum levels in CRC patients and patients with inflammatory bowel diseases

Two blood samples were taken from patients with advanced colorectal cancer, one before the start of chemotherapy and one after 3 months. CTC were analysed in both samples and CEA levels were determined. CTC were detected in 20/34 (59%) patients prior to chemotherapy (Table 1); 28/34 patients

Table 1 – Comparison of CTC with clinical outcome after therapy of colorectal cancer patients

Patient-ID	Therapy	Clinical validation	CTC		Serum CEA	
			Prior to therapy	Post therapy	Prior to therapy	Post therapy
2	5-FU/FA/CTP 11/bevacizumab	PD	–	–	+	+
9	5-FU/FA/CTP 11/bevacizumab	PD	+	+	+	+
11	5-FU/FA/oxaliplatin	PD	+	+	+	+
16	5-FU/FA/oxaliplatin	PD	–	–	+	+
18	5-FU/FA/CPT 11	PD	+	+	+	+
20	5-FU/FA	PD	+	–	+	+
23	5-FU/FA/mitomycin-C	PD	+	+	+	+
27	5-FU/FA/oxaliplatin	PD	–	–	+	+
32	5-FU/FA/oxaliplatin/cetuximab	PD	+	+	+	+
33	5-FU/FA/CPT 11	PD	–	–	+	+
5	5-FU/FA/oxaliplatin	SD	+	–	–	+
10	5-FU/FA/CPT 11/cetuximab	SD	–	–	+	+
13	5-FU/FA/oxaliplatin	SD	–	+	+	+
17	Capecitabin/CPT 11	SD	+	–	+	+
19	5-FU/FA/CPT 11/cetuximab	SD	+	+	+	+
28	5-FU/FA/oxaliplatin	SD	–	–	–	–
29	5-FU/FA/oxaliplatin	SD	–	–	+	+
31	5-FU/FA/oxaliplatin/bevacizumab	SD	–	–	+	+
4	5-FU/FA/oxaliplatin/bevacizumab	R	+	+	+	+
7	5-FU/FA/oxaliplatin	R	+	–	–	–
12	5-FU/FA/CTP 11	R	+	–	+	+
14	5-FU/FA/oxaliplatin/bevacizumab	R	+	–	+	+
15	5-FU/FA/oxaliplatin/bevacizumab	R	+	–	+	+
21	5-FU/FA/oxaliplatin/bevacizumab	R	+	+	+	+
22	5-FU/FA/oxaliplatin/bevacizumab	R	+	–	+	+
24	5-FU/FA	R	–	–	–	–
25	5-FU/FA/oxaliplatin/bevacizumab	R	–	–	+	+
26	5-FU/FA/oxaliplatin	R	–	–	+	–
30	5-FU/FA/CPT 11/cetuximab	R	+	–	+	–

5-FU, 5-flourouracil; FA, folinic acid; CTP 11, irinotecan-HCl; PD, progression; SD, stable disease; R, remission; +, positive; –, negative.

(82%) showed positive CEA serum levels; 17/20 patients (85%) with detectable CTC also showed elevated CEA serum; 14/34 patients (41%) were negative for CTC prior to therapy, elevated CEA values were detected in 11 of these patients (79%); 5/34 patients dropped out.

After 3 months of chemotherapy CTC were still detected in 9/29 patients (31%); 24/29 patients showed elevated CEA serum levels (83%) and 5/29 patients were negative for CEA serum levels (17%). CEA levels were increased in all patients in which CTC were still detectable after 3 months of chemotherapy. No CTC were detectable in 20/29 patients after chemotherapeutic treatment. In some patients, CTC seem to decrease under therapy in contrast to CEA serum levels which remain unaffected. Therefore, CTC may be more suitable for monitoring a therapy response.

To determine the specificity of the assay used we also analysed peripheral blood from patients with chronic inflammatory bowel disease. Therefore, peripheral blood from patients with Crohn's disease ($n = 32$) and ulcerative colitis ($n = 32$) was analysed. CTC were detected in 7/32 patients with Crohn's disease as well as 8/32 patients with ulcerative colitis. None of these patients developed colorectal cancer 30 months after the assay was performed. No correlation between the detection of CTC and disease activity was observed. However, since inflammatory bowel diseases are regarded as possible pre-cancerous the potentiality to evolve a tumour

remains. All patients with Crohn's disease and ulcerative colitis were negative for CEA serum levels (data not shown). This is in concordance with the expression of CEA on the mRNA level. Expression of CEA could be detected in only one patient with Crohn's disease. The specificity for the isolation and analyses of CTC in blood from healthy donors was >98% as described in Zieglschmid et al. (2007).

3.2. Detection of CTC in patients with advanced CRC before and after therapy

Blood samples of 34 patients with advanced CRC were analysed before therapy, 5 patients dropped out. Therefore, complete results of 29 patients were available (Table 1).

After therapy, 10/29 patients (34%) showed radiological tumour progression. CTC could be detected prior to and post therapy in 5/10 patients (50%) as shown in Figure 1 suggesting that detection of CTC might correlate with the progressive tumour burden. Only in 1/10 patients (10%) did the status change from CTC-positive prior to therapy to CTC-negative post chemotherapy. One possible explanation might be a change in the expression profile of the CTC in response to a therapy as described later. In 4/10 patients (40%) CTC could not be detected either before or after chemotherapy. In these patients it was not possible to make a statement about the response to therapy.

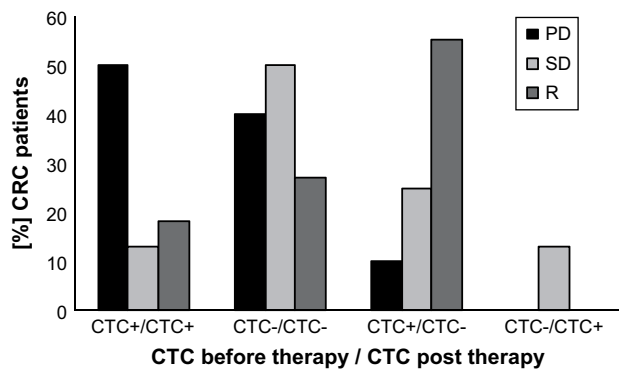


Figure 1 – Detection of CTC in CRC patients before and post therapy compared to clinical outcome. The analysis includes 29 patients. CTC were detected with AdnaTest ColonCancer and clinical outcome was determined with computer tomography. PD, progressive disease; SD, stable disease; R, remission; +, positive; –, negative.

Radiological remission was shown in 11/29 patients (38%). CTC were detected in peripheral blood from 6/11 patients (55%) prior to the start of chemotherapy but no CTC were detected in peripheral blood after chemotherapy indicating that the deletion of CTC might be a surrogate marker for response to chemotherapy. Only 2/11 (18%) patients were positive for CTC before beginning chemotherapy as well as 3 months after therapy. No CTC were isolated in 3/11 (27%) patients before and after initiation of chemotherapy.

Radiologically stable disease was shown in 8/29 (28%) patients. Different results were obtained in this group of patients. No CTC were detected in 4/8 patients (50%) before and 3 months after chemotherapeutic treatment, whereas CTC were still detectable after treatment in one patient. No CTC were found after treatment in 2/8 patients (25%), while no CTC were detectable in one patient before and after chemotherapy treatment.

The clinical relevance of CTC in the peripheral blood of patients with CRC (Cohen et al., 2006; Zieglschmid et al., 2007) and their usefulness as surrogate markers to monitor drug effects in metastasised breast cancer patients is an intense matter of debate (Hayes et al., 2006). Our data suggest that the analysis of CTC could be used as a surrogate marker to monitor tumour responses in patients with advanced colorectal cancer. In our study CTC were detected in peripheral blood from the majority of blood samples (59%) obtained prior to therapy. This number decreased to 31% in patients after treatment with chemotherapy. Similar results were obtained in patients with advanced breast cancer using the CellSearch system (Budd et al., 2006; Cristofanilli et al., 2005; Hayes et al., 2006). In contrast to the assay for the isolation and enumeration of CTC described in these studies we isolated CTC, analysed expression of tumour-associated transcripts by PCR and used this assay to investigate the efficacy of chemotherapies in advanced CRC patients.

3.3. Expression profiling of CTC in patients with advanced CRC before and post therapy

The expression profile of circulating tumour cells isolated from 20 different patients with colorectal cancer was

studied in more detail. Before chemotherapy was started the tumour-associated transcript CEA was expressed in 100% (20/20), EGFR in 10% (2/20) and GA733-2 in 35% (7/20) of all samples analysed (Figure 2). Patients treated with chemotherapy showed a decrease in CEA expression in 7/9 cases (78%), whereas the expression of EGFR and GA733-2 increased in 3/9 (33%) and 5/9 (56%) cases respectively. Further studies are needed to validate these findings and to verify whether the change in expression pattern is caused by the chemotherapeutic treatment. Interestingly, changing tumour cell expression patterns were also observed when we analysed CTC from patients with colorectal cancer before and after surgery of the primary tumour as well as from patients with advanced disease (Zieglschmid et al., 2007). At the time of the first diagnosis CTC from patients with colorectal cancer showed high EGFR expression and a low detection rate of CEA. On the other hand, CTC from patients with advanced disease revealed dominant CEA expression and low expression of EGFR. Contrary to the assumption that changes in the expression pattern of CTC could be caused by chemotherapeutic treatment, a different explanation might be a phenomenon called epithelial-mesenchymal transition (EMT) which occurs during the development of metastases. Before invading the blood circulation, tumour cells have to detach from the epithelial cell structure. One way to achieve that is that the tumour cell or the CTC respectively changes its expression profile to a more mesenchymal phenotype. This procedure is often related to a down regulation of epithelial cell markers e.g. E-cadherin and an up regulation of mesenchymal cell markers e.g. N-cadherin (Kang and Massague, 2004). As soon as the target organ is reached the CTC reverses the expression profile via mesenchymal-epithelial transition (MET) and evades the circulation. Since EGFR is a tumour-associated epithelial cell marker it may be another protein that underlies such changes in expression.

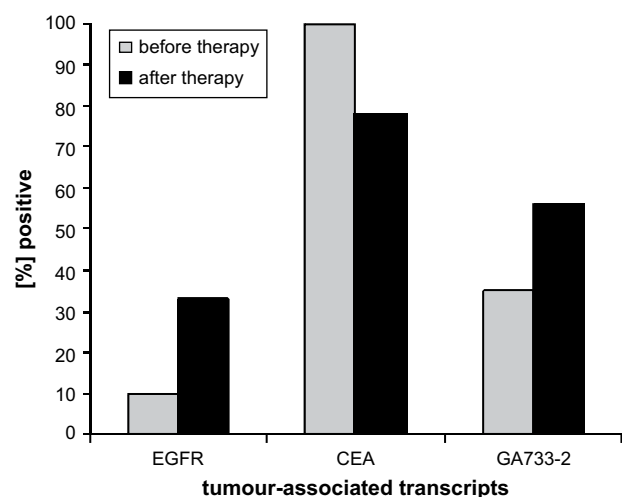


Figure 2 – Expression profile of CTC positive CRC patients before and after therapy. The analysis includes CTC positive patients determined with AdnaTest ColonCancer.

Table 2 – Detection of EGFR variants

EGFR variants	Crohn's disease (n = 32) (%)	Ulcerative colitis (n = 32) (%)	Colorectal carcinoma before therapy (n = 34) (%)	Colorectal carcinoma after therapy (n = 29) (%)
EX12_14del	3 (9)	1 (3)	3 (9)	–
EX12_15del	–	–	1 (3)	–

3.4. Detection of extracellular EGFR variants in CRC patients and patients with inflammatory bowel diseases

In our previous study we demonstrated a specificity of 100% for the detection of EGFR variants on CTC isolated from blood (Lankiewicz et al., in press).

We now extended our studies and tested peripheral blood obtained from patients with chronic inflammatory bowel disease, since it could be possible that inflammation associated circulating epithelial cells can be detected by the assay used in this study. cDNA from blood of patients with Crohn's disease (n = 32), ulcerative colitis (n = 32) and CRC patients before (n = 34) and after therapy (n = 29) was analysed for EGFR variants by RT-PCR with two primer pairs to detect different extracellular isotypes (Table 2). Three patients with Crohn's disease and one patient with ulcerative colitis expressed the EGFR variant EX12_14del. A typical analysis is shown in Figure 3. None of these patients developed colon cancer within a period of 30 months. However, since inflammatory bowel diseases are regarded as possible pre-cancerous the potential for a tumour to evolve remains.

Before initiation of chemotherapy circulating tumour cells from three patients with colorectal cancer expressed the EGFR variant EX12_14del and one of these patients also expressed the variant EX12_15del. After therapy no expression of EGFR variants could be detected in 2 patients (Figure 3). The third patient dropped out. The remaining 2 patients had radiologically stable disease, which was confirmed by the lack of CTC detection (Table 1). Potentially, CTC with an expression of

EGFR variants were eliminated by the administered therapies. For CRC patients it seems that the predominant mutation concerning EGFR is variant EX12_14del. This is in contrast to advanced breast cancer patients in whom the predominant mutation seems to be EX12_15del (Lankiewicz, 2006; Lankiewicz and Fehm, 2007). Further variants of the extracellular domain, especially EX2_7del (EGFR VIII), could not be detected either in patients with inflammatory bowel diseases or in CRC patients. This is in agreement with previous studies where no EGFR EX2_7del could be found in CRC patients (Azuma et al., 2006; Spindler et al., 2006).

The expression of EGFR variants in CRC patients seems to be tumour specific (Cunningham et al., 2005) as it is also described for glioblastoma, breast cancer, prostate cancer and NSCLC (Frederick et al., 2000; Ge et al., 2002; Ji et al., 2006; Moscatello et al., 1995; Olapade-Olaopa et al., 2000; Wikstrand et al., 1995). Variants have never been detected in the blood of healthy donors before but results of further experiments examining tumour entities like breast or lung cancer may be shown if the present results can be confirmed in general.

In summary, our results indicate that the detection of CTC in patients with advanced CRC could become a new tool to predict response to chemotherapy, and moreover, might provide an early opportunity to change therapy concepts. Since our findings are related to a relatively small group of patients, further studies with larger numbers of advanced CRC patients would be helpful to confirm the present data. One study might focus on the patients' treatment and how specific medication might have an influence on CTC and their expression pattern. Another

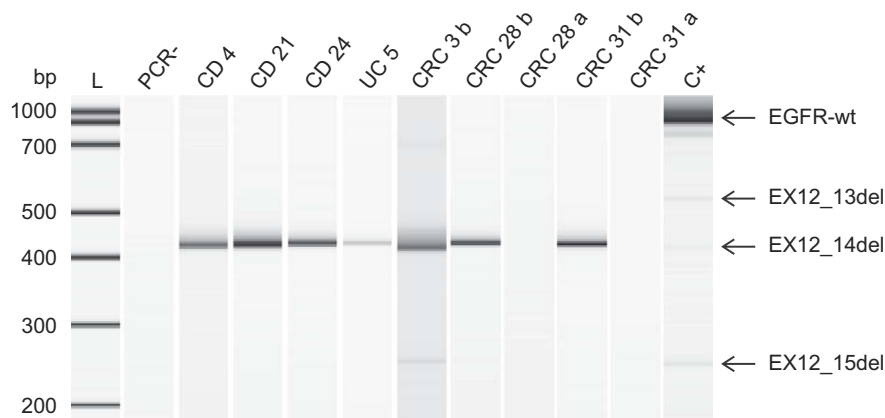


Figure 3 – Detection of EGFR variants in CRC patients, patients with Crohn's disease and ulcerative colitis. Amplified cDNA fragments of EGFR wild-type and different variants were analyzed by capillary electrophoresis with the Bioanalyzer 2100 and the DNA 1000 assay (Agilent Technologies, Böblingen, Germany). For positive control cDNA of a colorectal cancer cell line (T84) was used (Lankiewicz et al., in press). L, ladder; bp, base pairs; PCR–, negative control of the PCR; C+, positive control of the PCR; CD, Crohn's disease; UC, ulcerative colitis; CRC, colorectal cancer patients, the numbers represent the patients' ID; a, after therapy; b, before therapy; wt, wild-type. Patient CRC 3b dropped out before completing therapy.

study's objective could be to analyse the outcome of EGFR targeted therapies regarding EGFR variants on a larger scale.

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