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Monitoring of Circulating Tumor Cells by a Combination of Immunomagnetic Enrichment and RT-PCR in Colorectal Cancer Patients Undergoing Surgery*

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A – research concept and design; **B** – collection and/or assembly of data; **C** – data analysis and interpretation; **D** – writing the article; **E** – critical revision of the article; **F** – final approval of article

Abstract

Background. The presence of circulating tumor cells (CTC) has been reported in patients with advanced colorectal cancer. Monitoring CTC (also known as a liquid-biopsy) has recently become the center of interest for low-invasive monitoring of cancer progression and predictive biomarkers testing. Along with high-cost technology and a complex methodology, a straightforward method based on magnetic beads enrichment followed by RT-PCR is set to allow for routine CTC analysis in colorectal cancer patients.
Objectives. The main purpose of this study was to evaluate the possibility of CTC detection in routine monitoring of patients starting before and continuing after surgery.
Material and Methods. The investigated group consisted of 30 patients mainly in advanced stages of colorectal cancer. In all patients, CTC detection was performed prior to surgery, in a subset of 14 patients additional sampling was done during and after surgery. In all cases, peripheral blood was processed using AdnaTest ColonCancer kit, which relies on enriching CTCs using EpCAM-functionalized magnetic beads and subsequently identifying tumor-specific CEA, EGFR and GA733-2 mRNA transcripts.
Results. Out of all the tested samples, CTC were found in one patient suffering from advanced disease with lung and liver metastases. There, however, the positive finding was confirmed in 3 consecutive samples acquired before, during and shortly after palliative R2 resection.
Conclusions. The presence of CTC may be used to observe post-operative disease development. Due to the overall low CTC detection, further technology development may be necessary before its universal applicability to manage colorectal cancer patients (**Adv Clin Exp Med 2016, 25, 6, 1273–1279**).

Key words: CTC, colorectal cancer, CEA, EGFR, GA733-2.

Colorectal cancer is one of the most common causes of death among malignant diseases in developed countries. Metastases detected at the time of diagnosis or occurring as a result of progression of previously treated tumors lead to 90% of deaths [1, 2].

Metastasis is a multistep process in which malignant cells detach from the primary tumor tissue

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to establish a secondary deposit on a remote location. It is known that 1 g of primary tumor tissue releases approximately 1×10^6 malignant cells into the blood stream per day [3]. At the same time, only 0.01% of the circulating tumor cells (CTC) are capable of metastasizing [4]. The main prerequisite is sufficient vascularization of the primary tumor, the lack of malignant cell adhesion, and their release into the lymphatic or blood vessels. In the circulation system, a large part of CTC is destructed by mechanical factors as well as specific and nonspecific immune responses. When reaching the distant organ site, CTC adhere specifically to the target endothelium following a change of cell phenotype and the clonal expansion of secondary tumor deposits, metastases. The presence of CTC in peripheral blood was first described by Asworth in 1869 [5]. Progress in the detection of CTC was achieved by immunohistochemistry testing [6] and, more recently, by highly sensitive PCR techniques [7–9]. In patients with colorectal cancer, the presence of CTC in peripheral blood was repeatedly confirmed and their detection ranged from 20% for localized disease and 60% in advanced CRC [10–18]. The clinical relevance of CTC as an independent prognostic factor was documented for cases in patients with positive CTC at diagnosis who had poorer prognosis than patients with negative CTC [10, 12–14, 16–24].

Currently, there are several approaches to detecting CTC in colorectal cancer noted in the literature (Table 1) [25]. Due to the low concentration of CTC in peripheral blood (one CTC in 106 to 107 leukocytes), CTC detection methods are frequently performed in two steps. The first step is CTC "enrichment" by either centrifugation in a density gradient, by means of porous membrane filtration or immunomagnetic separation through the establishment of cell surface antigens on the magnetic particles and the subsequent separation in a magnetic field. Some of them utilize enrichment on magnetic beads coated with anti-EpCAM (Epithelial Cellular Adhesion Molecule) antibody [12–14, 16–18]. EpCAM, a transmembrane glycoprotein, is an adhesion molecule expressed on most epithelial cells. Its main function is in molecular adhesion and, as such, is frequently overexpressed on cancer cells, especially during the process of migration and metastasis [26]. The second step is the detection of CTC by immunocytochemical cell sorting or by highly sensitive molecular genetic methods such as RT-PCR (reverse transcription PCR) directed at tumor-specific mRNA. Interestingly, some authors have performed RT-PCR even without prior pre-concentration [10, 27]. Aside from just detecting their presence, the molecular profile of CTC may serve as a predictor of treatment response and survival as demonstrated in several studies [11, 12, 28–30]. The technique known as the "liquid biopsy" has recently generated a strong interest among molecular oncologists as a viable alternative in cases where tissue sampling is not available [31].

The enriched EpCAM-positive cells may then be subjected to either direct detection by fluorescence labelling and flow cytometry [13, 14, 17, 18]

Table 1. Overview of approaches for detection of CTC

Title	Principle of enrichment	Principle of detection	References
AdnaGen Test	immunomagnetic separation (EPCAM)	molecular analyses (in CRC detection mRNA GA 733-2, CEA, EGFR)	Zieglschmid, 2006, Lankiewicz, 2008; Cristofanilli, 2009, Hauch, 2007; Fehm, 2009; Tewes, 2009
CellSearch system	immunomagnetic separation (EPCAM)	negative selection by CD-45; Positive selection by CK-8, 18, 19	Sastre, 2007; Cohen, 2008, 2009; Matsusaka, 2011; Botteri 2010
MACS system	immunomagnetic separation (EPCAM)	immunolabelling, FISH, mRT-PCR	Serrano, 2009
CEE Microfluidics	antibody functionalized surfaces with a microfluidics channel	immunolabelling, FISH, mRT-PCR	Mayer, 2011
MagSweeper technology	immunomagnetic separation (EPCAM)	immunolabelling, FISH, mRT-PCR	Talasaz, 2009
MAINTRAC analysis	immunomagnetic separation (EPCAM)	laser scanning cytometry	Pachmann, 2005
CTC Chip	EpCAM coated microposts under strict manipulation of velocity and shear force	negative selection by CD-45; Positive selection by CK and further molecular and genetic analyses	Nagrath, 2007; Uhr, 2007; Stott, 2010

or indirect identification by tumor-specific RT-PCR [12, 16, 32–35]. In the alternative approach, CTCs are identified by RT-PCR performed directly on the genetic material isolated from plasma without prior magnetic enrichment or with the assistance of cell sorting [10, 22, 23, 30, 35] or cell capturing on a microfluidic device [32–34]. There are two major commercial systems utilizing the above approaches. Although both systems are certified for *in vitro* CTC diagnostic: CellSearch (Veridex) and AdnaTest (AdnaGen), reports on their routine use in clinical environment are still scarce. In this paper we will present our results of CTC detection in patients with colorectal cancer undergoing surgical treatment. In the prospective study we used the AdnaTest kit with two-step protocol including immunomagnetic enrichment in the first step followed by RT-PCR of tumor-specific mRNA transcripts epidermal growth factor receptor (EGFR), carcioembryonic antigen (CEA) and gastrointestinal tumor-associated antigen 2 (GA733-2). We present data for frequency and a clinical utility of CTC monitoring to observe the clinical course of the disease in association with the operation.

Material and Methods

The prospective study included 30 patients scheduled for a surgical treatment of colorectal cancer. There were 10 women and 20 men aged between 47 and 90 with the median of 47 years (75.6 years in women and 70.4 years in men). The primary tumor localization was in the sigmoideum and rectum (×17), in transversum (×5), in descendens (×5) and in caecum or ascendens (×3). Most patients were in stage 3 or 4 of the disease (11 and 9 cases, respectively), 7 patients were in stage 2, and 3 patients were in stage 1. All patients signed informed consent to their participation in the study. The initial sampling was performed before the planned surgery. In patients undergoing neoadjuvant therapy, the sampling was scheduled at least 5 days following completion of the last chemotherapy cycle prior to surgery. In some patients, subsequent samples were also acquired during the surgery and on the 1st and 7th day after the surgery. All CTC experiments were performed by a two-part AdnaTest ColonCancer kit (AdnaGen, Langenhagen, Germany) using original protocols. Total of 5 mL of peripheral blood was drawn into AdnaCollect Blood Collection Tubes (AdnaGen) containing proprietary dedicated cell-stabilization solution. Samples were transferred to the laboratory and processed within 24 h from the sampling in accordance with the instructions. The magnetic enrichment was performed using the AdnaTest ColonCancerSelect part of the kit, the subsequent RT-PCR was completed with AdnaTest ColonCancerDetect part. Amplified PCR products were detected by Agilent Bioanalyzer 2100 on-chip capillary electrophoresis instrument (Agilent Technologies, Waldbronn, Germany).

Results

Out of the 30 tested, we have recorded 1 patient with the presence of CTC. The patient was in stage 4 of rectal cancer with generalization of the disease to liver and lungs. This patient was a 64-year-old male who, at the time of the sample acquisition, was diagnosed with generalized colorectal cancer with lung and liver metastases. The persistence of CTC immediately after the operation is consistent with the R2 resection (palliative sigmoideostomy) followed by palliative biological targeted therapy by bevacizumab. Somewhat surprisingly, CTCs were not detected in any of the other 8 patients with metastatic disease, including 2 patients with confirmed metastatic spread to both liver and lungs. Hence, the overall rate of CTC detection was 11% in the group of patients in stage 4, and 3% in the overall colorectal cancer group. As shown in Fig. 1, one patient maintained CTC-positivity during 3 consecutive samplings including blood taken prior to surgery, the second sample taken during the surgery (palliative sigmoideostomy) and the third sample taken on the 1st day after the surgery in the occurrence of EGFR as well as CEA mRNA transcripts. All CTC positive samples from this patient revealed the presence of CEA mRNA transcripts. The first sample taken prior to the surgery also shoved EGFR mRNA. Finally, the sample taken on the 7th day as well as the last sample taken 3 months after the surgery were both CTC-negative. In total, 61 samples were acquired and tested for the CTC presence. The overview of results is shown in Table 2.

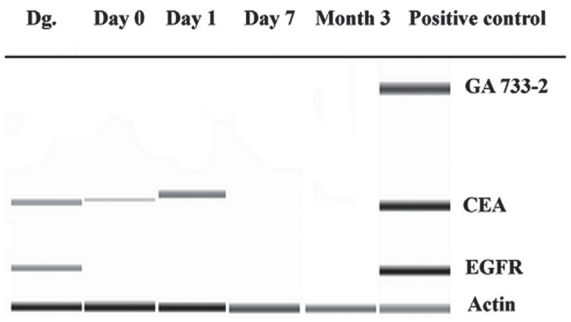

Fig. 1. A Bioanalyzer gel image of results obtained from patient number 17. The CTC were detected through CEA marker positivity in 3 consecutive samples taken prior to surgery (Dg.), during the surgery (Day 0), and 24 h after the surgery (Day 1).

Table 2. Overview of patients in the study with CTC data

Patient	Primary tumor location	Stage	RT-PCR positivity/CTC status				
			before surgery	during surgery	1st day	7th day	3 months
1	sigmoid colon	II	b-a/n				
2	rectum	II	b-a/n				
3	rectum	IV	b-a/n				
4	transverse colon	III	b-a/n				
5	rectum	I	b-a/n				
6	descending colon	III	b-a/n				
7	rectum	III	b-a/n				
8	transverse colon	II	b-a/n				
9	rectum	III	b-a/n				
10	ascending colon	III	b-a/n				
11	cecum	III	b-a/n				
12	transverse colon	II	b-a/n				
13	descending colon	III	b-a/n				
14	transverse colon	III	b-a/n				
15	rectum	IV	b-a/n	b-a/n	b-a/n	b-a/n	
16	rectum	IV	b-a/n		b-a/n	b-a/n	
17	rectum	IV	beta-actin, CEA, EGFR/positive	beta-actin, CEA/positive	beta-actin, CEA/positive	b-a/n	b-a/n
18	descending colon	IV	b-a/n		b-a/n	b-a/n	
19	rectum	II	b-a/n	b-a/n	b-a/n		
20	rectum	IV	b-a/n	b-a/n	b-a/n	b-a/n	
21	rectum	IV	b-a/n				
22	rectum	III	b-a/n				
23	rectum	I	b-a/n		b-a/n		
24	rectum	I	b-a/n	b-a/n	b-a/n	b-a/n	
25	rectum	III	b-a/n	b-a/n	b-a/n		
26	rectum	IV	b-a/n	b-a/n	b-a/n		
27	rectum	II	b-a/n	b-a/n	b-a/n		
28	ascending colon	II	b-a/n	b-a/n	b-a/n		
29	transverse colon	IV	b-a/n	b-a/n	b-a/n		
30	sigmoid colon	III	b-a/n	b-a/n	b-a/n		

b-a/n – beta-actin/negative.

Discussion

The AdnaTest ColonCancer IVD CE kit by AdnaGen is based on a two-step protocol. The stabilized blood is first mixed with suspension of paramagnetic beads whose surface is functionalized with anti-EpCAM antibody. The second step includes extraction of total RNA followed by RT-PCR detection of tumor-specific mRNA transcripts CEA, EGFR and GA733-2. Although the

AdnaTest is not suited for CTC quantification, its sensitivity as claimed by the manufacturer is 2 tumor cells in 5 mL of blood [11, 12, 16]. In the previously published AdnaTest validation study, 20 out of 34 patients (58.8%) with advanced colorectal cancer showed the presence of CTC [12]. Detection rates at other clinical stages are not available in the literature. The study by Sieuwerts et al. [35] has shown that even some breast cancer cell lines express EpCAM in levels that are too low to allow capture using CellSearch. In our group of patients in various stages of the disease, only 1 patient out of 9 (11%) with an advanced disease has shown CTC. Although we have carefully followed the recommended procedure for both sample acquisition and next steps, CTC were observed in none of the 21 patients in stages 1 through 3. It is important to note that all samples from 30 patients have shown beta-actin housekeeping expression. This serves as an internal control confirming the presence of mRNA material in the sample (Table 2), effectively excluding a methodical error. Supported by numerous reports in the literature, the presence of CTC in colorectal patients (especially in advanced metastatic disease stages) can hardly be questioned [10–18]. The successful CTC recovery naturally relies on proper timing and methodology applied during the sample acquisition. In addition, we observed the far lower detection of CTC than the expected rate. It is also interesting that none of the three CTC positive samples (all from the same patient collected in defined time of disease) displayed mRNA transcripts from GA733-2, which actually codes for the EpCAM protein product [36]. The main possible cause of the low CTC detection could be the nonspecific leukocytes binding. The genetic material from nonspecifically bound hematopoietic cells, naturally expressing beta-actin, may saturate the RT-PCR reaction, thus impeding detection of EGFR, CAE and GA733-2 transcripts originating from CTCs. Based on microscopic detection or CD45 positivity, the contamination of CTC fraction by leukocytes was found [38–40]. Up to 100 leukocytes may be concurrently isolated with the tumor cells [38]. Contamination may be related to the character of immunobeads. When negative or positive immunobeads used alone as in our study, there was found a significant evidence of contamination from peripheral blood leukocytes which was minimized by combined method [39]. The Adnagen method does not specifically recognize leukocytes and, therefore, we cannot declare their presence or even quantification. Another potential explanation for this may be related to the inferior bind of CTC to anti-EpCAM antibodies, which is caused by hidden epitopes as a result of different conformational states of the cell surface EpCAM protein. Inferior binding was observed in the study by Antolovic et al., who compared two different specific antibodies against the epitopes in extracellular domain of EpCAM and showed that KS1/4 system retrieved 10 fold more CTC compared to the BerEP4 system [40]. Synergic effect of antibodies was detected. The combination of more antibodies resulted in a higher total recovery rate of tumor cells than would be assumed by addition of the recovery rates obtained with single antibodies [11]. AdnaGen included magnetic beads with two immobilized antibodies – BerEP4 and MOC31; thus, it could be supposed that applying the system with mixture of antibodies leads to more effective enrichment of CTCs. Another factor which may contribute to failed CTC capture is lower expression of EpCAM and other markers during an epithelial-to-mesenchymal transition (EMT) as part of an oncogenic pathway to increased invasiveness and metastatic potential [42], as a result of chemotherapy or treatment [43]. Thus, anti-EpCAM antibodies in AdnaGen system may not be efficient to capture CTCs.

In advanced colorectal cancer, circulating tumor cells represent a viable biomarker for observing the efficiency of clinical treatment of colorectal cancer. Its use for the detection of early signs of the disease relapse or anticancer therapy resistance is apparent. In our case, the low CTC detection rates are to be further evaluated. From the above (notices, comments) result, the system containing a mixture of more antibodies with higher specificity and affinity is necessary to better capture tumor cells and minimize the contamination by leukocytes.

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