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Circulating tumor cells in different stages of colorectal cancer

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Abstract

Introduction. Liquid biopsies are noninvasive tests using blood or body fluids to detect circulating tumor cells (CTCs) or the products of tumor cells, such as fragments of nucleic acids or proteins that are shed into biological fluids from primary tumor or its metastates. The analysis of published clinical studies provides coherent evidence that the presence of CTCs detected in peripheral blood is a strong prognostic factor in patients with colorectal carcinoma (CRC). The aim of the study was to implement size-based separation protocol of CTCs in CRC patients.

Material and methods. Patients diagnosed with different stages of CRC (n = 98) were included in the study. All patients have been diagnosed for colorectal adenocarcinoma by pathology examination, 45 patients with colon carcinoma and 53 with rectosigmoid cancer. A size-based separation method (MetaCell®) for viable CTC enrichment from peripheral blood was used to assess the presence of CTCs by cytomorphological evaluation using vital fluorescence microscopy.

Results. Cytomorphological analysis revealed that 81 (83%) tested samples were CTC-positive and 17 (17%) were CTC-negative. We report a successful isolation of CTCs with proliferation potential in patients with CRC. The CTCs were cultured *in vitro* for further downstream applications. Some of the isolated CTCs were able to grow *in vitro* for 6 months as a standard cell culture.

Conclusions. We established a reliable, inexpensive and relatively fast protocol for CTCs enrichment in CRC patients by means of vital fluorescence staining which enables their further analysis *in vitro*.

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Key words: circulating tumor cells; colon cancer; rectosigmoid cancer; staging; cell culture; MetaCell®

Introduction

An idea of a minimally invasive way to obtain accurate information on tumors from a blood sample, also known as liquid biopsy, has gained increasing attention in cancer diagnosis, risk stratification and monitoring treatment response. Liquid biopsies are noninvasive tests using blood or bodily fluids to detect circulating tumor cells (CTCs) or the products of tumor cells, such as fragments of nucleotides or proteins that are shed into biological fluids from primary or metastatic tumors [1]. The analysis of published clinical studies provides coherent evidence that the presence of CTCs detected in peripheral blood is a strong prognostic factor in patients with colorectal carcinoma (CRC) [1–3].

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The majority of the studies used immunomagnetic methods or RT-PCR for CTCs detection [1, 3–5]. These methods are dependent on specification of separated CTCs, especially, their cell surface antigens such as epithelial cell adhesion molecule (EPCAM) or they rely on a specific targeting of RNA sequences. This seems problematic for these methods because one of the key aspects that emerged from the analysis of CTCs is their remarkable heterogeneity, both considering the expression of specific cancer-associated markers, and also their phenotypic characteristics such as tumor-seeding potential. Therefore, we have taken a different approach based on the implementation of cell-size separation protocol of CTCs in CRC patients. This study presents first data of the application of this method to determine the occurrence of CTCs in the differently staged CRC patients.

Material and methods

Patients. To date 98 patients with diagnosed CRC have been enrolled into the study in accordance with the Declaration of Helsinki. All patients were candidates for surgery or surgical diagnosis. Based on the informed consent, clinical data were collected from all patients. For each patient, approximately 18 mL of venous blood was drawn from the antecubital vein and placed into S-Monovette tubes (Sarstedt AG & Co., Numbrecht, Germany) containing 1.6 mg EDTA/mL of blood as an anticoagulant. The samples were processed at room temperature using an isolation procedure completed within 24 hours after the blood draw.

CTCs enrichment and culture. A size-based separation method for viable CTC enrichment from peripheral blood has recently been introduced (MetaCell®, MetaCell s.r.o., Ostrava, Czech Republic) [6, 7]. The size-based enrichment process is based on the filtration of peripheral blood through a porous polycarbonate membrane (with pores of 8 μm diameter). The minimum and maximum volume of the filtered peripheral blood may be adjusted up to 50 mL with physiological fluid. The standard 8 mL peripheral blood sample from patients suffering from CRC was transferred into the filtration tube. Detailed separation protocol has been described previously [8–10]. The CTCs were grown in RPMI medium with 10% fetal blood serum (FBS, both purchased from Sigma-Aldrich, St. Louis, MO, USA) for a minimum of 3 days on the membrane. Alternatively, the enriched CTCs fraction can be transferred from the membrane and cultured directly on any plastic surface or a microscopic slide, or the separation membrane may be transferred on a microscopic slide. Microscopic slide is preferred if cytological analysis is planned. If an intermediate analysis of CTCs is awaited, the CTCs fraction is transferred in phosphate-buffered saline (PBS, 1.5 mL) to a cytospin slide. The slide is then dried for 24 h and analyzed by cytology (May-Grünwald staining) and/or by automated immunocytology protocols (Ventana, Benchmark Ultra, Roche, Tucson, AZ, USA) using standard differential diagnostic antibodies for the pathology evaluation process.

Cytomorphological analysis. After 3–5 days of culture, nucleus and cytoplasm of viable cells were stained by vital fluorescent dyes Nucblue® Reagent (Thermo Fisher Scientific, Waltham, MA, USA) and Celltracker™ Green CMFDA Dye (Thermo Fisher Scientific), respectively. Staining procedures followed manufacturer protocols.

The stained fixed cells captured on the membrane were examined using light microscopy in two steps: (i) screening at ×20/×40 magnification to locate the cells; (ii) observation at ×40/×60 magnification for detailed cytomorphological analysis. Isolated cells and/or clusters of cells of interest (immunostained or not) were selected, and their digital images were then examined by an experienced researcher and/or pathologist. CTCs were defined as cells with the following characteristics: (i) with a nuclear size ≥ 10 μm); (ii) irregular nuclear contour; (iii) visible cytoplasm, cells size over 15 μm; (iv) prominent nucleoli; (v) high nuclear-cytoplasmic ratio; (vi) presence of proliferating cell, (vii) actively invading cells creating 2D or 3D cell groups.

Cell cultures of CTCs obtained from colorectal cancer patients. Membrane with captured cells was washed with RPMI medium and transferred into culture plate. Four mL of RPMI medium supplemented with 10% FBS, amphotericin B (Sigma-Aldrich) and penicillin-streptomycin (Sigma-Aldrich) were added to the culture medium. Captured cells were cultured *in vitro* under standard conditions (37°C, 5% CO_2) for 3–5 days.

Statistical analysis. The Chi-Square test was used to determine if there is a significant relationship between two nominal (categorical) variables (*e.g.* metastasis presence and CTC-positivity). Contingency tables in order to decide whether or not effects are present were analyzed (http://vassarstats.net/newcs.html). Statistical significance of differences between two compared groups and CTC presence was set at $p < 0.05$.

Results

Patients' characteristics

Patients diagnosed with different stages of CRC (n = 98, 61 male and 37 female patients) were included in the study (Table 1). The median age was 61.7 years (range 38–90 years). All patients were diagnosed with colorectal adenocarcinoma by pathology examination, 45 patients presented with

Table 1. Clinical-pathological characteristics of the colorectal cancer patients' cohort and the occurrence of circulating tumor cells (CTCs) in blood

Patients	**n**	**Positive CTC**	**%**	**Negative CTC**	**%**	**p**
Colon cancer	45	40	88.89	5	11.11	n.s.
Rectosigmoid cancer	53	41	77.36	12	22.64	n.s.
Tumor size		Positive CTC		Negative CTC		
T1	6	5	83.33	1	16.67	n.s.
T2	19	13	68.42	6	31.58	n.s.
T3	52	44	84.61	8	15.38	n.s.
T4	21	19	90.48	2	9.52	n.s.
Lymph node involvement		Positive CTC		Negative CTC		
N0	52	43	82.69	9	17.31	n.s.
N1	20	17	85.00	3	15.00	n.s.
N2	26	21	80.77	5	19.23	n.s.
Metastasis		Positive CTC		Negative CTC		
M0	67	54	80.60	13	19.40	n.s.
M1	31	27	87.10	4	12.90	n.s.
Grade		Positive CTC		Negative CTC		
G1	3	3	100.00	0	0	n.s.
G2	63	50	79.37	13	20.63	n.s.
G3	32	28	87.50	4	12.50	n.s.
Stage		Positive CTC		Negative CTC		
I	18	14	77.78	4	22.22	n.s.
II	28	26	92.86	2	7.14	n.s.
III	18	13	72.22	5	27.78	n.s.
IV	33	28	84.85	5	15.15	n.s.

The characteristics of the patients' cohort and CTC occurrence was compared by chi- square test. At $p \geq 0.05$ the comparison showed no significant (n.s.) difference for the tested subgroups.

colon carcinoma and 53 with rectosigmoid cancer (Table 1).

Cytomorphological analysis revealed that 81 (83%) of all CRC patients were CTC-positive in peripheral blood. Into study were enrolled 45 patients with colon cancer of which 40 (89%) were CTC-positive and 53 patients with rectosigmoid cancer of which 41 (77%) patients were CTC-positive (Table 1).

The presence of CTCs in different stages of colorectal cancer according to TNM classification

The frequency of the CTC positivity is reported for different patient subgroups in relationship to tumor size, lymph node involvement and distant metastases. The CTC positivity for different disease stages and tumor grade subgroups is summarized in Table 1. Patients were CTC positive in majority of all TNM stages, and disease and grade stages (Table 1).

Cultures of CTCs isolated from blood of colorectal cancer patients

We report a successful isolation of CTCs with proliferation potential in patients with CRC. The cells captured by size-based filtration approach showed a viable condition, which enabled setting up cultures of CTCs which viability was unaffected by antibodies (*e.g.* anti-EPCAM antibodies) or lysing solutions (*e.g.* erythrocyte lysing solution). The CTCs were cultured *in vitro* for further downstream applications (Fig. 1). Some of the isolated CTCs were grown *in vitro* for as long as 6 months as a standard cell culture.

Discussion

The classification of tumor (pTNM staging) and the residual tumor status following treatment are the strongest predictors of outcome for patients with CRC. A careful pTNM classification enables an accurate estimation of prognosis; therefore, it can be

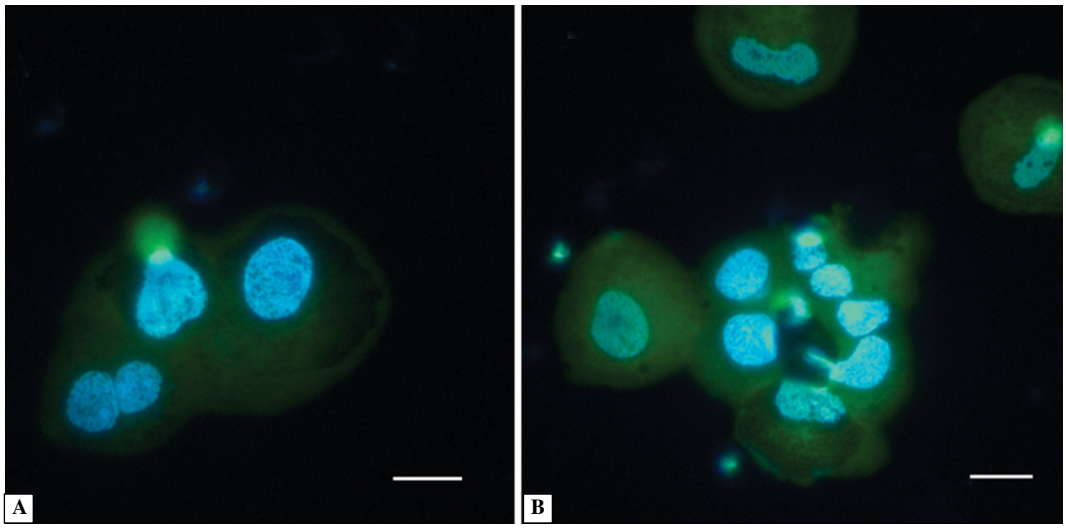

Figure 1. Morphology of CTCs isolated from patients with CRC. **A.** CTCs captured on the separation membrane; **B.** CTCs after successful short term *in vitro* cultivation — 5 days after isolation. Cells were visualized by vital fluorescent staining with NucBlue® and Celltracker® as described in Material and methods. Bars represent 10 μm.

considered the gold standard for analyzing the results of any treatment [11].

Similarly, CTCs enumeration has been established as a prognostic marker for CRC [1, 12–14]. In a large meta-analysis including 12 studies between 1998 and 2011, the presence of CTCs in patients with metastatic colorectal cancer (mCRC) correlated with shorter progression-free survival (PFS) and overall survival (OS) [14].

Matsusaka *et al.* showed that patients with ≥ 3 CTCs in 7.5 mL blood at 2 and 8–12 weeks after initiation of chemotherapy had shorter median progression-free survival (PFS) and overall survival (OS) than patients with < 3 CTC counts. Patients with CTC counts ≥ 3 at baseline with a decrease in the CTC count to < 3 during treatment had a median PFS that was similar to patients with persistently low CTC counts. They concluded that a decrease in CTC count to < 3 at 2 weeks after initiating chemotherapy was an indicator of treatment efficacy [15].

The use of different methodologies for CTC detection has shown conflicting results, and the lack of a standardized technology complicates the implementation of CTCs examination in routine clinical practice. In addition, significant differences in CTC detection rates among the molecular methods of their detection have been reported [16–18].

In comparison with other studies [1–3, 12–15] the detection rate of CTCs in the present study seems high. However, a recent study of breast cancer showed that tumor cells can leave the primary site very early during tumor progression and evolve independently at the metastatic site [19, 20]. The genetic analyses showed that 80% of metastases were derived from early disseminated tumor cells [20].

CTCs can be used for longitudinal molecular and genetic analyses of the tumor and may aid in targeted therapy investigations. CRC patients with CTCs carrying wild-type KRAS show longer PFS and OS when treated with chemotherapeutics and cetuximab [21]. The detection of KRAS mutation in CTCs from peripheral blood may predict the response to cetuximab plus chemotherapy in CRC patients [21]. These findings indicate that the detection of KRAS mutational status in CTCs by using gene expression array has potential clinical applications for selecting CRC patients who may benefit from cetuximab therapy.

CTCs detection and characterization may be a valuable tool to refine prognosis and CTCs can be predictive biomarkers in treatment of colorectal cancer.

Here we presented high accordance of the TNM stages of CRC with the occurrence of CTCs in patient's blood detected by a reliable, inexpensive and relatively fast method for CTCs enrichment and evaluation based on cytomorphological analysis by means of vital fluorescence staining.

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