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Significance of Cytokeratin Fragment M65 and Cytokines IL6, IL8 and IL17A in Bone Marrow Aspirates of Colorectal Cancer Patients

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Research Article

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ABSTRACT

Aims: Soluble cytokeratin (CK) fragments and inflammatory interleukins (ILs) in bone marrow (BM) aspirates of colorectal cancer (CRC) patients are expected to indicate presence of disseminated tumor cells (DTCs) and anticancer response of the host, respectively. The present study investigated the relations of CK18 fragment M65, IL6, IL8, and IL17A in BM samples to the presence of DTCs and prognosis.
Place and Duration of Study: Department of Medicine (Medical Unit II) and Department of Surgery, Donauspital Vienna, between 2002 and July 2005.
Methodology: BM aspirates were obtained immediately prior to and one and two years after tumor surgery, respectively, and M65 and cytokines were quantified by ELISA assays.
Results: 16/66 patients revealed tumor-positive BM aspirates, and 10/46 evaluable patients relapsed within five years. M65 levels exhibited no relation to either positive biopsies, relapses or methylation status of O^6-methyl guanine methyl transferase (MGMT). In contrast, IL17A concentrations of BM aspirates were elevated in non-relapsed versus relapsed, as well as MGMT-wildtype versus MGMT-methylated patients. Due to large individual variations, IL6 and IL8 levels of BM showed no significant differences for non-relapsed versus relapsed patients.
Conclusion: M65 levels of BM samples of CRC patients exhibited no correlation with micrometastases or disease recurrence, respectively; however, patients who achieved disease-free survival revealed increases of IL17A in BM aspirates, possibly indicating immune response to tumor cells.

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Keywords: Colorectal cancer; bone marrow; disseminated tumor cells; cytokeratin; M65; IL6; IL8; IL17; MGMT;

1. INTRODUCTION

Although significant progress has been made in the therapy of colorectal cancer (CRC), patients who underwent apparently curative surgery reveal a considerable number of tumor relapses and, therefore, a subpopulation of high-risk patients may benefit from adjuvant chemotherapy (Gollins, 2010). Currently available prognostic tests have not enough reliability to select for patients at risk for tumor progression (Rousseau et al., 2010). However, development of superior biomarkers may lead to identification of patients who are likely to benefit from cytotoxic therapy and optimization of treatment schemes with improved outcomes (Cunningham et al., 2010). Dissemination of tumor cells from primary tumors into the circulatory system is an early event during carcinogenesis and significant numbers of disseminated tumor cells (DTCs), as detectable in the peripheral blood or BM aspirates by different methods, might represent an indicator of early relapses and a poor prognosis (Lin et al., 2011; Pantel and Alix-Panabières, 2010). In breast cancer, the presence of DTCs in BM was predictive for the development of distant metastases, and persistence of these cells in BM was an independent prognostic factor for survival (Braun et al., 2005). In addition, there is some evidence that the detection of DTCs in BM of prostate cancer patients may represent a prognostic parameter (Riethdorf et al., 2008).

However, the results for CRC are less clear, and positive as well as negative evidence was reported for an association between DTCs in BM and increased recurrence rate or reduced survival, respectively (Steinert et al., 2008; Peach et al., 2010; O'Connor et al., 2005). Presence of circulating tumor cells (CTCs) in peripheral blood at least 24 h after resection of colorectal tumors was published to represent an independent prognostic marker of recurrence (Peach et al., 2010). In addition to technical difficulties in detection of DTCs, it is not clear which fraction of cells with a specific phenotype are capable of inducing tumor relapses (11). Therefore, several studies were conducted to elucidate the clinical relevance of DTCs in CRC presenting a very heterogeneous picture varying in dependence of different patient groups, sample sizes, follow-up times, target antigens and staining methods, all of which probably account for the observed variation in DTC detection rates in association with clinical parameters (Riethdorf et al., 2008). Our own results revealed that presence of DTCs in BM of CRC patients was variable over the course of disease and lacked prognostic significance (Buxhofer-Ausch et al., 2010).

Detection of soluble cytokeratin (CK) fragments released from epithelial tumor cells may serve as an alternative method using a biochemical marker to confirm the presence of residual tumor cells in CRC patients (Schlimok et al., 1990). CK18 is an intracellular, mainly insoluble protein highly expressed by various types of epithelial cells. A caspase-cleaved 30 kD and a 65 kD fragment of CK18 are released into the extracellular compartment during apoptosis and necrosis, respectively, though export of intact CKs has also been reported (Alix-Panabières et al., 2008). These fragments can be quantified by the M30-Apoptosense® ELISA detecting CK18Asp396-NE M30 neo-epitope and the M65® assay (Peviva, Bromma, Sweden), respectively (Linder et al., 2004). Previously, we demonstrated that perioperative alterations of M65 and M30 levels in serum samples of CRC patients correlated with positive BM biopsies and early tumor relapses, respectively (Ausch et al., 2009a; Ausch et al.,

2009b). In the present study we investigated whether detection of CK18 fragments in BM samples of the same CRC patients may possibly improve diagnosis of micrometastasis.

Furthermore, detection of selected inflammatory cytokines was expected to reflect an immune response of patients to DTCs. It was therefore investigated additionally, whether interleukins (ILs), such as IL6, IL8 and IL17A, in BM correlated with elevated levels of M65 and the occurrence of DTCs. IL8 is known to promote angiogenesis and increase growth, survival as well as migration of cancer cells (Waugh and Wilson, 2008). Elevated blood levels of IL6, IL8 and IL17 were reported for defective mismatch repair colorectal cancer (Le Gouvello et al., 2008). IL17 induces the production of other cytokines, including IL6, IL8 and others, from several cell types (Pages et al., 1999). In conclusion, the aim of the present study was to investigate a correlation of the levels of IL6, IL8, IL17A and the CK18 fragment M65 in BM aspirates either to biopsy status, promoter methylation of MGMT and prognosis. MGMT repairs guanine bases in DNA that were methylated incorrectly and is frequently silenced in colorectal tumors (Nagasaka et al., 2003). Furthermore, MGMT promoter methylation was associated with increased base pair transitions, high frequency of KRAS mutations and microsatellite instability (Span et al., 1996).

2. MATERIAL AND METHODS

2.1 Patients

The study population consisted of 66 colorectal cancer patients. Of these patients, 45 were male, 21 female, median age was 68.2 years (range 45 – 81 years) and tumor stages I in 18 cases, stage IIA/B in 12 cases, stage IIIA/B/C in 19 cases, stage IV in 11 cases and six patients with tumor relapses were included. All patients were treated at the Danube Hospital, Vienna, Austria, between 2002-2005. None of the patients had received chemo- and/or radiotherapy before. A control group consisted of 14 patients biopsied for diagnosis of hematological diseases and proved to be free of malignancy. The study was approved by the ethics committee responsible for Vienna municipal hospitals.

2.2 Blood and BM Samples

Blood samples were taken preoperatively, and one and two years after surgery, centrifuged at 2,000 rpm for 10 min and supernatants stored at -80°C. BM aspirates were obtained from both upper iliac crests by needle aspiration (2x5 ml). Preoperative aspirations were performed under general anesthesia shortly before surgery and follow-up aspirations under local anesthesia. Mononuclear cells were isolated by Ficoll-Hypaque density gradient centrifugation and supernatants stored at -80°.

2.3 Detection of DTCs

For immunocytochemical staining of DTCs, the monoclonal antibody A45-B/B3 (Micromet, Munich, Germany) directed against a pan-CK epitope was used (Buxhofer-Ausch et al., 2010). Detection was accomplished with Idetect Super Stain System Fast Red (ID Labs, London, ON, Canada). All slides were independently examined by two blinded observers. A minimum of one tumor cell per $2x10^6$ screened mononuclear cells containing clearly discernible nuclei was interpreted as a positive result and indicative of DTCs in BM.

2.4 Determination of M65, IL6, IL8 and IL17A

M65 in serum and BM samples was quantified using the M65® ELISA assay (Peviva, Bromma, Sweden), and cytokines in BM samples were determined by the Human IL6, IL8 and IL17A ELISA MAX™ Standard Kits (Biolegend, San Diego, CA, USA) according to the manufacturer´s instructions, respectively.

2.5 Determination of Methylation of the MGMT Promoter

DNA was extracted using a commercially available DNA extraction kit (QIAmp DNA Mini Kit; Qiagen, Hilden, Germany) and quantified by fluorometry (Quant-iT dsDNA HS Assay; Invitrogen, Carlsbad, CA, USA). The sodium bisulfite treatment was carried out using the EZ DNA Methylation Kit (Zymo Research, Anopoli Medical Systems, Eichgraben, Austria). RT-PCR was performed using primers specific for methylated or unmethylated alleles, respectively. Primer sequences were as follows: for methylated reactions, forward 5´ TTTCGACGTTCGTAGGTTTTCG 3´, reverse 5´ GCACTCTTCCGAAAACGAAACG 3´; for unmethylated reactions, forward 5´ TTTGTGTTTTGATGTTTGTAGGTTTTTGT 3´, reverse 5´ AACTCCACACTCTTCCAA AAACAAAACA 3´. RT-PCR conditions were as follows: 95°C for 15 min, then 30 cycles of 95°C for 30 s, 62°C for 30 s and 72°C for 30 s, and finally 10 min at 72°C. RT-PCR products were separated on 2% agarose gels, stained with ethidium bromide and visualized under UV light.

2.6 Statistics

Values are demonstrated as mean ± SD. Statistical analysis was performed using Student's t-test. Differences with *$p < 0.05$ were regarded as statistically significant.

3. RESULTS AND DISCUSSION

16/66 CRC patients revealed tumor-positive BM aspirates, and 10/46 evaluable patients relapsed within a follow-up period of five years. For a subgroup of 30 CRC patients, levels of CK fragment M65 were compared for serum samples and BM aspirates collected shortly before tumor surgery. A comparison of M65 concentrations in peripheral serum samples with BM aspirates revealed good correlation with a correlation coefficient of $R^2 = 0.87$, $p < 0.001$ (Figure 1).

The mean M65 concentration in BM aspirates of cancer patients at all three time points was 300 ± 125 U/l and proved to be elevated compared to a control group consisting of 14 patients biopsied for diagnosis of hematological diseases and found to be tumor-free revealing a significantly lower mean BM M65 concentration of 185 ± 18.7 U/l. However, M65 concentrations of BM aspirates of CRC patients collected shortly before surgery as well as one and two years during follow-up showed no significant differences for relapsed and disease-free patients over a five-year period (Figure 2).

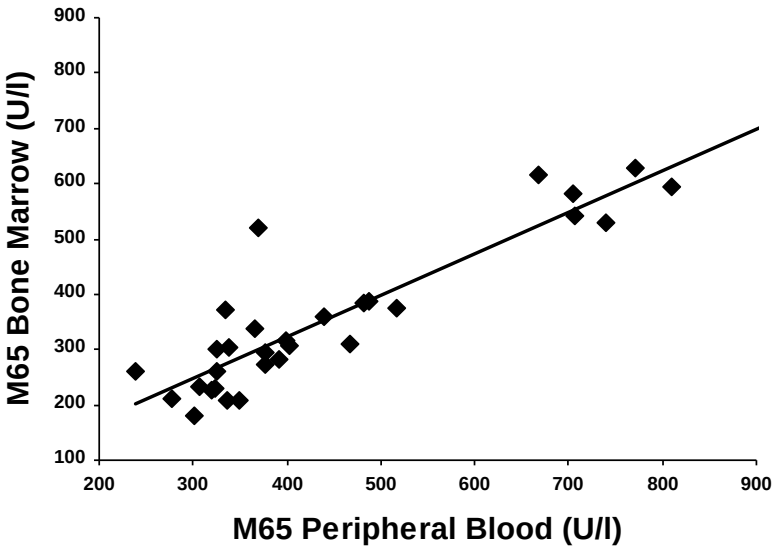

Fig. 1. Comparison of M65 levels in serum and BM

M65 levels were determined in serum and BM samples derived from 32 CRC patients before surgery. Correlation of peripheral and BM concentrations of M65 was analyzed by linear regression (r^2= 0.87, p < 0.01).

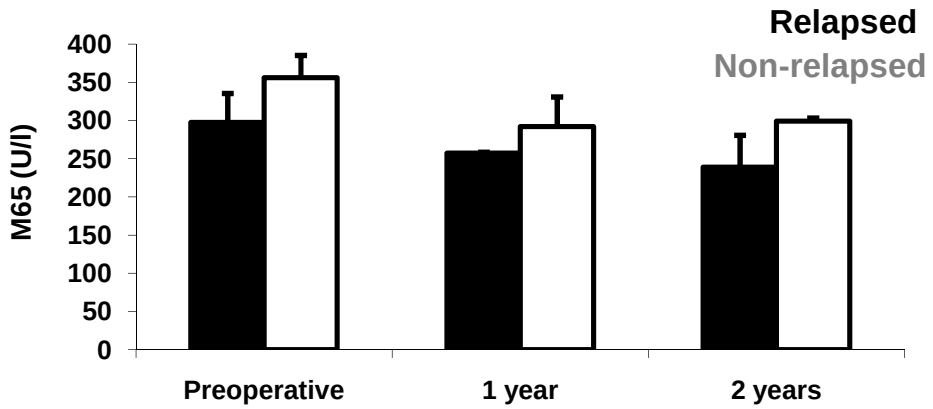

Fig. 2. M65 levels in BM and relapse of disease

M65 was assessed shortly before, one year and two years after surgery, respectively. Data are presented as mean ± S.E.M. All differences were nonsignificant.

In order to assess presence of minimal residual disease, BM aspirates were collected shortly before surgery and during follow-up after one and two years after removal of the tumors. DTCs were detected in the mononuclear cell fraction of BM samples of 16/66 patients using the A45-B/B3 pan-CK antibody for immunohistochemical staining. Analysis of the M65 levels

of the same aspirates showed no quantitative differences between DTC-positive and – negative patients (Figure 3).

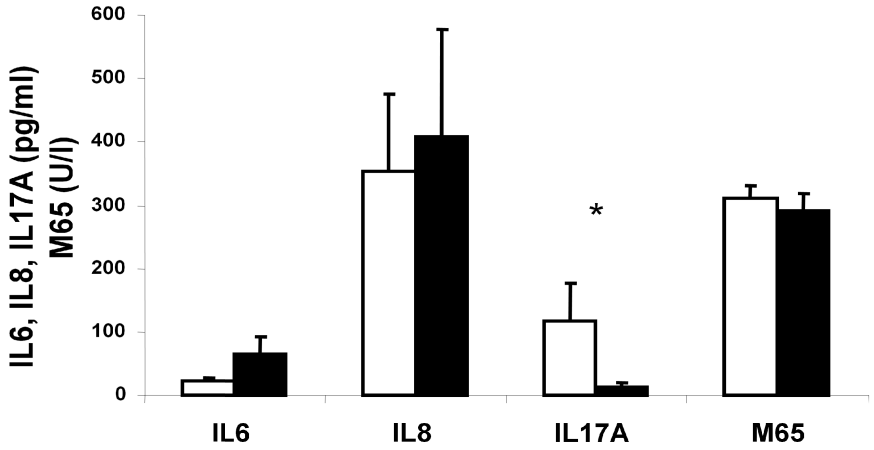

Fig. 3. Correlation of cytokine and M65 levels with DTC

*Cytokine (IL6, IL8, IL17A) and M65 levels were determined in 50 biopsy-negative (white column) and 16 biopsy-positive (black column) BM samples derived from CRC patients shortly before and one and two years after surgery, respectively. Data are presented as mean ± S.E.M. With the exception of IL17A (*p = 0.045), all differences were nonsignificant.*

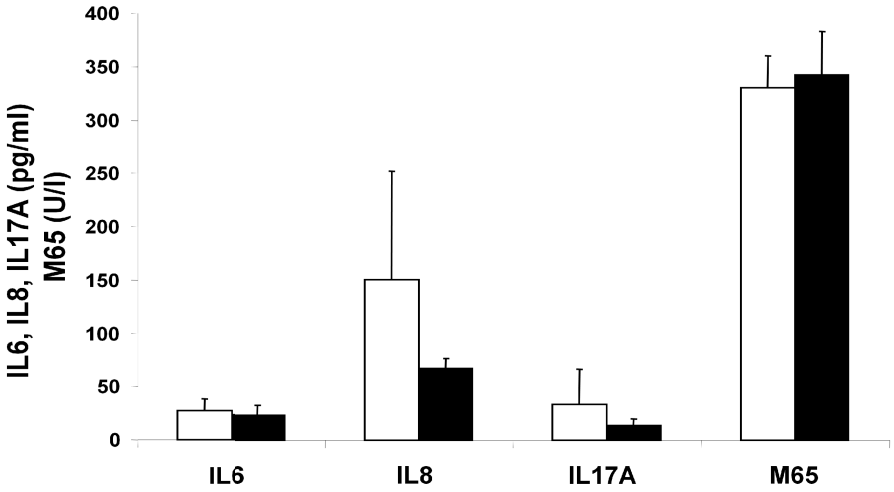

Fig. 4. MGMT status and BM cytokines

Methylation status of the MGMT promoter region was assessed by methylation-specific PCR. Data are presented as mean ± S.E.M. All differences between cytokines and M65 for patients with active (white columns) or silenced (black columns) MGMT were not statistically significant.

In order to detect potential changes of cytokine expression in BM in response to DTCs, specimens were used to measure IL-6, IL-8 and IL-17A. Levels of IL6 and IL8 in BM aspirates exhibited large intra- and interindividual variations, and with the exception of IL17A, which was elevated in BM of biopsy-negative patients, no significant differences between DTC-positive and -negative samples were revealed (Figure 3). Following stratification of the patients according to their MGMT methylation status, an important determining factor for KRAS mutation and prognosis, the same cytokines were evaluated (Figure 4).

31/57 evaluable patients showed an unmethylated promoter region and 26 methylated promoter region of MGMT, respectively. Again, IL6 and IL8 were not significantly different for active or silenced MGMT, respectively, and IL17A was elevated in patients who exhibited a nonmethylated MGMT promoter region; however, the difference did not reach statistical significance. Similarly, M65 levels in BM were not different for patients with divergent MGMT status. Tumor progression after apparently curative resection of CRC is caused by tumor cell dissemination, currently undetected by standard clinical staging procedures. The detection of DTCs in lymph nodes, blood or BM by RT-PCR or immunohistochemistry could help to identify a subgroup of patients at risk for disease relapse who may thus benefit from adjuvant therapy.

Although judged at low risk, 10-20% of patients with CRC stages I and II actually will develop recurrent disease (Liefers et al., 1998). In this regard, several studies demonstrated that detection of DTCs was clearly related to early relapse and decreased survival of the respective patients in several tumor entities, especially for breast cancer. Whereas CRC rarely metastasizes to bone, this compartment may function as a reservoir of tumor cells or presence of tumor cells in BM may indicate micrometastasis to other organs (Pantel and Alix-Panabières, 2010; Roth et al., 2009). Results regarding a possible significance of DTCs in CRC remain controversial (Sastre et al., 2008). Clinical follow-up studies showed a significantly higher relapse rate in CRC patients presenting with CK-positive cells in their BM at time of primary surgery (Schlimok et al, 1990). In contrast, the presence of epithelial cells in the BM was detected in approximately two thirds of 140 CRC patients who underwent curative surgery, but did not correlate with tumor stage or survival (Steinert et al., 2008). Furthermore, multivariate analysis failed to identify occult tumor cell dissemination into any body compartment as an independent prognostic factor of disease-free survival. The presence of DTCs in BM of CRC patients detected by RT-PCR, but not immunohistochemistry, did predict a worse clinical outcome after removal of liver metastases, pointing to the significance of the respective method for detection of DTCs (Vogelaar et al., 2010).

RT-PCR products for CK8 and CK18 were demonstrated in all cancer cell lines of gastrointestinal origin, but variable amounts of several CKs were found to be amplified from control BM specimens from non-cancer patients (Dimmler et al., 2001). Most CK18 molecules form insoluble filaments inside the cell, but a pool of soluble CK18 (M65), released into the extracellular compartment by intact cells or during cell death, can also be demonstrated (Riethdorf et al., 2008; Chou et al., 1993). We found that perioperative changes of the peripheral blood levels of CK fragments in CRC patients seem to indicate presence of tumor micrometastasis associated with worse prognosis (Ausch et al, 2009a; Ausch et al., 2009b; Ausch et al., 2010). Comparison of preoperative M65 levels in serum with BM aspirates of CRC patients in the present study revealed good correlation and, therefore, it may not be necessary to resort to BM aspirates. However, all M65 BM concentrations measured at the three time points showed no significant differences between

samples obtained from DTC-positive and –negative as well as relapsed and disease-free patients. Although M65 levels were higher than in a tumor-free control group, this parameter is not suitable for identification of high-risk patients with residual tumor load. These results corroborate findings obtained by ELISA determination of CK19 in BM samples revealing significantly elevated levels in cancer patients (Höchtlen-Vollmar et al., 1997). In good accordance, this assay gave a larger number of positive results compared to immunohistochemistry, but did not correlate with TNM stage or histological grading. Similarly, M65 BM concentrations were not different in patients with inactivated MGMT compared to MGMT-expressing tumors, although methylation of the promoter region of MGMT characterizes a subpopulation of patients with mutations in KRAS and thus poor prognosis (Span et al., 1996).

Chronic inflammation precedes or accompanies a substantial number of cancers (Gonda et al., 2009). Homing of tumor cells in the BM niche may possibly provoke an immune response mediated by inflammatory cytokines. However, determination of IL6 and IL8 in our BM samples revealed large variability for the different time points and patients. Their levels were not significantly different in patients with DTC-positive and –negative BM or different status of MGMT methylation, respectively. Although IL6 and IL8 play important roles in tumor cell growth, bone metastasis and cancer progression, their production, either by or in response to colorectal DTCs, may be obscured by nonmalignant processes (Waugh and Wilson, 2008; Ara and Declerk, 2010; Meads et al., 2008).

On the contrary, IL17A levels in BM were significantly elevated in DTC-negative compared to DTC-positive patients. IL17 and IL17-releasing cells were shown to be implicated in inflammation and immune response (Xu and Cao, 2010). IL17 is believed to be mainly produced by T helper 17 (Th17) cells, a unique subset of T helper cells different from Th1 and Th2 cells. Basically it acts as proinflammatory cytokine and induces the release of certain chemokines, other cytokines, matrix metalloproteinases and antimicrobial peptides from mesenchymal and myeloid cells. Although Th17 cells were found in an experimental animal cancer model as well as in human cancers, it remains controversial whether these cells promote tumor growth or regulate antitumor responses (Ji and Zhang, 2010). IL17 was furthermore reported to play a potential role in T cell-mediated angiogenesis and in promotion of tumorigenicity in human cervical cancer (Tartour et al., 1999). Th17 cells producing IL17A were locally accumulated in early disease of gastric cancer and decreased upon progression (Maruyama et al., 2010). Additionally, infiltrating Th17 cells were associated with increased survival in ovarian cancer patients (Kryczek et al., 2009; Radosavljevic et al., 2010). In CRC patients IL17 serum levels were significantly higher than in control subjects. In contrast, other findings proved that IL17 was undetectable in approximately half of CRC patients, and no significant difference was observed regarding IL17 levels between CRC and matched normal tissue or between plasma samples of patients and healthy control subjects. Immunohistochemistry of tumor samples revealed heterogenous immunoreactivity of IL17 in 65% of the cases which was interpreted as a minor or partial role of IL17 in CRC (Wägsäter et al., 2006). On the contrary, tumor-selective activation and cytotoxic activity of $CD8^+$ tumor infiltrating lymphocytes and tumor-selective migration of $CD4^+$ T helper cells were demonstrated in colorectal cancer and their numbers linked to an improved prognosis in colorectal cancer (Koch et al., 2006).

Furthermore, we used methylation-specific RT-PCR to determine the methylation status of the MGMT promoter region in DNA extracted from CRC patients who had undergone surgery. Promoter methylation of this gene was found in a high percentage of cases (46%). CRC patients with unmethylated MGMT promoters were much more likely to experience

recurrence of the disease within 36 months than patients with hypermethylated MGMT promoters (Nagasaka et al., 2003). However, these patient groups showed no differences in BM concentrations of all cytokines tested and M65.

4. CONCLUSION

- In summary in the present study, M65 levels of BM samples of CRC patients exhibited no correlation with the presence of micrometastases or disease recurrence, respectively. However, patients showing disease-free survival for a follow-up of five years revealed increases of IL17A in BM aspirates indicating a putative immune response to the DTCs.
- Quantitation of M65 in serum and BM samples of CRC patients revealed good correlation; however, M65 BM levels exhibited no correlation with the occurrence of DTCs in BM or relapse of the disease, respectively.

- Concentrations of IL6 and IL8 were not different for patients revealing negative or positive DTC status, respectively, but BM levels of IL17A were higher in samples of DTC-negative compared to DTC-positive patients, which may indicate active immune surveillance and elimination of tumor cells.

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