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Article type: Original article

Received: January 10, 2020.

Accepted: February 12, 2020.

Published online: February 20, 2020.

ISSN: 1897-9483

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The impact of bone marrow disseminated tumor cells on survival and disease progression in left-sided colorectal cancer patients.

Authors:

Radoslaw Pach¹, Katarzyna Dylag-Trojanowska², Jaroslaw Baran³, Antoni Czupryna¹, Maciej Siedlar³, Justyna Zybaczynska⁴, Philip H. Brandt⁵, Antoni M. Szczepanik¹

¹ First Department of General, Oncological and Gastroenterological Surgery, Jagiellonian University Medical College, Cracow, Poland

² Department of General and Oncological Surgery, John Gawlik Hospital, Sucha Beskidzka, Poland

³ Department of Clinical Immunology, Institute of Pediatrics, Jagiellonian University Medical College, Cracow, Poland

⁴ Jagiellonian University Medical College, Cracow, Poland

⁵ Baystate Medical Center-Springfield, Massachusetts, United States of America

Short title: Prognostic value of bone marrow DTC in left-sided colorectal cancer.

Corresponding author: Antoni Szczepanik PhD, e-mail: antoni.szczepanik@uj.edu.pl, telephone: +48 12 4002400, First Department of General, Oncological and Gastroenterological Surgery, Jagiellonian University Medical College, 30-688 Cracow, Poland, Jakubowskiego street 2.

Conflict of interest: none declared.
What’s new?

The study shows that there were statistically significantly less metachronous distant metastases in patients with left-sided colorectal carcinoma and disseminated tumor cells (DTC) in bone marrow than in those without DTC in bone marrow. DTC were surprisingly not a negative predictive factor of distant metastases and not a negative prognostic factor in our study group. This result underpins the notion that there are substantial differences between colorectal cancer in different locations. As a result, further studies should address the issue and a tailored approach should be established for patients with this neoplasm.
Abstract

Introduction
Disseminated tumor cells (DTC) are the subset of circulating tumor cells that migrated to the bone marrow. Colorectal cancer is a heterogeneous disease according to the location of the primary tumor.

Objectives
The aim of this study was to analyze the relation between the presence of DTC in the bone marrow and tumor characteristics and long-term treatment results in left-sided colorectal cancer.

Patients and methods
A prospective analysis of 91 left-sided colorectal cancer patients (37 with colon cancer and 54 with rectal cancer) treated between 2007 and 2012 in one tertiary center was carried out. The study included patients with following cancer stage: I-15, II-26, III-26 and IV-24 patients. Overall survival and cancer relapses were compared in patients with different cancer stages and DCT status.

Results
The DTC in bone marrow were diagnosed in 42 patients (46.1%). The prevalence of DTC in the bone marrow was not related to tumor infiltration depth, nodal involvement, distant metastasis, tumor grading nor the location of the primary tumor. The 5-year overall survival in the DTC positive group was 59.5% while for DTC negative patients 53% (p = 0.190). There was a visible trend favoring survival in patients with DTC in the bone marrow when patients with stage II and III disease were analyzed together and separately for stage II and III. There were significantly less metachronous distant metastases in DCT positive patients.

Conclusions
The presence of DTC in the bone marrow is not associated with primary tumor characteristics and seems to diminish metastasis formation in left-sided colorectal cancer. There is also a trend for improved overall survival for DCT positive patients. These results are provocative and warrant further confirmation.
Keywords

circulating tumor cells; distant metastases; left-sided colon cancer; overall survival;
prognostic biomarker;

Abbreviations

DTC – disseminated tumor cells, BM – bone marrow, CRC – colorectal cancer, SD – standard deviation, CK – cytokeratins
Introduction

The significance of disseminated tumor cells (DTC) in the bone marrow of colorectal cancer patients is not clear. DTC are identified within the bone marrow in 17-64% of CRC patients (median 29%). [1] They are a subset of circulating tumor cells that migrate to the bone marrow and can form micrometastases. This phenomenon is mostly described in breast cancer patients. Although DTC in bone marrow occurs in a substantial percentage of cancer patients without distant or nodal metastases a large portion of them will not experience distant metastases in the future. Additionally, cancer cells in the bone marrow may be dormant for several years before re-entering circulation however such situations are very uncommon in colorectal cancer. Two meta-analyses of studies on the prognostic significance of circulating cancer cells in colorectal cancer indicated that the presence of DTC in the peripheral blood is a negative prognostic factor. [2,3]

At present, there is insufficient evidence that the presence of DTC in the bone marrow of colorectal cancer patients influences the prognosis. [4,5,6,7] Such studies, especially in non-disseminated colorectal cancer (CRC), require a large number of patients and a long follow-up period as the median survival in radically operated colorectal cancer patients is longer than 5 years. In addition, colorectal cancer is not a homogenous disease and depending on the location of primary tumor it's understood that right-sided and left-sided cancers may have disparate biology and different prognosis. [8,9,10,11] Therefore this study concentrated on malignancies arising from the left colon and rectum, with locally advanced cancer being the main area of interest.
The aim of this study was to analyze the relationship between the presence of DTC in bone marrow with the tumor characteristics, cancer progression and survival in left-sided colorectal cancer.

Patients and Methods

The study involved a group of 91 colorectal cancer patients treated in a single institution between 2007 and 2012. Only patients with tumors located in the rectum or left colon were included. The term left colon was defined as a section of colon distal to the left 1/3 of the transverse colon. The inclusion criteria were age over 18, histologically confirmed cancer and electively performed surgical procedure. Patients with synchronous right-sided colon cancer or patients with a history of other neoplastic diseases were excluded. None of the colon cancer patients received preoperative chemotherapy, while 5 of the rectal cancer patients received preoperative radiotherapy and 2 preoperative chemoradiotherapy. The group was composed of 42 women and 49 men with the mean age of 64.7 (SD 10.2). Cancer stage in analyzed patients was the following: T1-2 pts, T2-16 pts, T3-60 pts, T4-13 pts, N0-42 pts, N1-20 pts, N2-25 pts, Nx-4 pts, M0-67 pts, M1-24 pts. The clinicopathological characteristics of the patients are summarized in Table 1.

A bone marrow biopsy from the posterior superior iliac spine was performed on the day of surgery after the induction of general anesthesia. A 5ml sample of the bone marrow was collected into plastic tubes containing EDTA (ethylenediaminetetraacetic acid).

Pelleted cells from bone marrow samples were incubated with an excessive amount of lysing solution (Becton Dickinson Biosciences, San Jose, CA, USA) for 10 min, repeated 3–4 times to remove erythrocytes. The remaining cells were washed in phosphate-buffered saline (PBS) and adjusted to the concentration of 1 × 10^7 cells ml⁻¹ in PBS. Subsequently, the cells were
stained with monoclonal mouse anti-human CD45 (phycoerythrin labeled) antibodies (DAKO, Glostrup, Denmark) and sorted into CD45⁺ and CD45⁻ populations using flow cytometry (FACS Vantage SE, BD Biosciences, Bedford, MA, USA) equipped with the TurboSort (BD Biosciences) option and Aerosol Protection System (Flexoduct International ApS, Greve, Denmark). The Innova Enterprise II ion laser (Coherent, Santa Clara, CA, USA) operating at 488 nm was used as a light source. Sorting was performed using a 70 mm nozzle tip with a drop drive frequency of 65 kHz, 1.5-drop envelopes and a ‘normal’ sorting mode. Sorted CD45⁻ cells were collected into polystyrene Falcon 2057 tubes (BD Biosciences) precoated with fetal calf serum and maintained in a refrigerated bath recirculator (Neslab Instruments, Portsmouth, NH, USA). About 1 × 10⁶ of CD45⁻ cells (1 × 10⁶ cells ml⁻¹) were used to prepare slides. The slides were dried, fixed with a mixture of ethanol and acetone (1 : 1 v v⁻¹), and then stained for 30 min with A45-B/B3 monoclonal antibodies (5 μg ml⁻¹) (Micromet GmbH, Germany), which recognize common epitopes of cytokeratins (CK) including CK 8, 18 and 19. Subsequently, the slides were washed and stained for 30 min with goat anti-mouse IgG-FITC-labelled antibodies (DAKO). After washing with PBS, the slides were assayed within 2 days. The CK⁺ cells were identified by two independent investigators under a BX60 fluorescent microscope (Olympus, Tokyo, Japan) and documented with a DP10 camera (Olympus). At least 300 cells were examined per slide. The samples were regarded as positive when at least three CK⁺ cells were found per slide. Accordingly, patients were classified into CK⁺ and CK⁻ groups. All CK+ cells were cytologically malignant cells as seen under the microscope. We could not distinguish between different cytokeratins. On the other hand, the antibody (A45-B/B3) was commonly used in a number of studies about DTC in the bone marrow, so the comparison with other research is possible. Cytokeratins 8, 18 and 19 are the epitopes of normal colonic mucosa, colonic adenoma, and adenocarcinoma. Therefore,
the staining of the tumor for these epitopes is not routinely used and was not performed in analyzed patients.

The surgical procedures were carried out according to oncological guidelines. Due to the changes in the TNM staging systems during the study period, all the specimens were re-staged according to the TNM 7th edition. The clinical and pathological data were recorded. Patients received postoperative chemotherapy if indicated regardless of their DTC status. All patients were followed up for at least 5 years, or until death, and dates of death were verified by the census registry office.

All the patients provided their informed, written consent. The study was approved by the Jagiellonian University Ethical Committee KBET no 86/B/2007 and KBET no 122.6120.128.2015. The study was registered at ClinicalTrials.gov, registration number NCT03640572.

The statistical analysis was conducted with Statistica 13 software (StatSoft Inc.). The distribution of variables was checked by Kolmogorov-Smirnoff test. The categorical variables were compared with Chi-square test with Yates’ correction. Survival analysis was performed according to Kaplan Meier method and log-rank test. The value of p<0.05 was established as statistically significant.

Results

CK positive cells were identified in the bone marrow of 42 patients (46.1%); 16 in the left colon (43.2%), and 26 of the patient’s with rectal cancer (48.1%), without a statistically significant difference (Table 2). The prevalence of DTC was not related to the depth of
infiltration (T feature) and was similar in T1-2 and T4 patients. There was no statistically significant difference between the prevalence of DTC in N- and N+ patients. Patients with and without distant metastasis also had similar rates of DTC detection in the bone marrow. The prevalence of DTC in the bone marrow of patients with TNM stages I-IV was similar, with a slightly lower prevalence in Stage IV patients. The presence of DTC in the bone marrow was not related to either the tumor grade or the resection radicality.

The number of patients who received preoperative treatment was low, however, 5 of the rectal cancer patients received preoperative radiotherapy, and 3 of those had DTC in their bone marrow. Two rectal cancer patients received preoperative chemoradiotherapy and five weeks after treatment completion both of them were found to harbor DTC in their bone marrow.

All the patients were followed-up for at least five years. The 5-year survival rate for the DTC positive patients was 59.5%, while for the DTC negative patients it was 53%. The difference between groups was not statistically significant. (Figure1). Because DTC could, in theory, be a source of distant metastasis and cancer progression, further analysis of patients in stages II and III was performed. Stage IV patients were excluded because they already had disseminated disease, while Stage I patients were excluded because of a very low probability of tumor progression. In the group with stage II and III patients, the 5-year survival rates were 59.2% and 76% for DTC negative and positive patients respectively (Figure 2a). Even though the difference was almost 17% in favor of DTC positive patients, it was not statistically significant. The proportion of stages II and III in DTC negative and positive groups was 14/13 and 12/13 respectively. The mean age of DTC negative patients was 63 years while in DTC positive group 65.3 years. The trend towards the better survival of DTC positive patients was also marked in stages II and III separately, however, due to the small number of patients, it did not reach statistical significance (Figure 2b,2c).
Disease recurrence was assessed in stages II and III. Metastatic disease was diagnosed in 13 patients (25%). Sites of metastasis were varied, with the prevalence of liver metastasis (7 of 13 patients). In the DTC negative group, 10 cases were observed, while in the DTC positive group only 3 cases were observed. The difference between the occurrence of distant metastasis between the mentioned above groups was found to be statistically significant (p=0.0375).

Discussion

This study was started in 2007 and the authors decided to use methodology already established for gastric cancer in colorectal cancer patients. [12] The preliminary results were not published earlier as such a study requires long term follow up. At the current time point, all the patients recruited were followed for at least five years. The group of patients in this investigation was more homogenous than in other studies since only left-sided colon and rectal cancer patients were included. Most studies involved populations of colorectal cancer patients that had both left and right-sided disease or only included patients with rectal cancer. Right-sided colon cancer has different clinical and molecular characteristics than left-sided colon and rectal cancer and it’s proposed to analyze it separately in clinical and scientific studies. [13]

The DTC in bone marrow were diagnosed in 46.1% of patients. The other studies reported an incidence between 10 and 63.6% [7,14] although, the majority of studies reported it at the level between 25-40%. With the high number of epitopes of the tumor cells used and different methods of its detection (immunocytochemistry, immunomagnetic assay, RTPCR - reverse-transcriptase polymerase chain reaction) there is no standard method for identifying DTC
within the bone marrow. [15] The rate of detection is different with regard to the method used, even in the same study. [16,17] The method which was used within the presented study is based on FACS (fluorescence-activated cell sorting) cell sorting and the detection of cells expressing the common epitope of cytokeratins 8,18 and 19 within the sorted population. Therefore, this method may be more sensitive than other simpler methods as the cell population is enriched with the initial sorting.

The incidence of DTC in the bone marrow was not related to the depth of tumor infiltration, nodal involvement, nor distant metastasis. Similar observations were characterized in a German study of colon cancer patients. [16] However, in the aforementioned group of patients, over 40% had tumors located in the right-sided colon. Others supported these findings in different groups of colorectal patients, [4,7] but there were also contrary observations. [18,19] There is also a common opinion that the tumor grade does not influence the rate of bone marrow DTC. [4,16]

We observed no difference in the prevalence of DTC between left colon and rectal cancer patients. This is in line with other studies that demonstrated similar results. [4,20] For colon cancer patients, neoadjuvant therapy is the exception and not the norm, but rectal cancer patients may receive preoperative radiotherapy or chemoradiotherapy according to indications. In our study, only 7 rectal cancer patients underwent preoperative treatment. While this represents a low number, this method was not used routinely in the first study period. It was not anticipated that the short course radiotherapy would affect the DTC, however, 5 weeks of chemoradiotherapy could potentially eradicate such cells. The study on DTC in rectal cancer showed that they are identified less frequently in patients who responded to preoperative chemoradiotherapy. [21] Moreover, in a study on gastric cancer, chemotherapy was able to significantly reduce the prevalence of DTC in the bone marrow. [22] We observed only two patients who received preoperative chemoradiotherapy. After this therapy, both of
them were DTC positive. This observation has no statistical power but clarifies that the preoperative treatment did not bias results in rectal cancer patients.

The overall survival at 5 years in the DTC positive group was 59.5% while for DTC negative patients it was 53%. The difference was statistically not significant, and both groups had comparable stages according to TNM classification. This observation alone was not astonishing since other studies demonstrated that the presence of DTC in bone marrow may not be a negative prognostic factor for colorectal cancer patients. This was also the conclusion of one of the meta-analyses published in 2010, [23] however authors paid attention to the fact that the methods of DTC detection were inhomogeneous. Therefore, there is still a place for new studies that enrich the population of patients diagnosed using a specific method. For further analysis, we excluded the most advanced stage IV patients. The prognostic significance of bone marrow DTC was described in stage IV colorectal cancer, however only in patients after curative resection of liver metastasis. [24] On the other hand, others demonstrated that this phenomenon may be related to the method of detection used. [17] We could not find a study that demonstrated the prognostic significance of DTC in Stage IV colorectal cancer patients not treated with the radical resection of metastasis. In our Stage IV patients, only one had a simultaneous liver resection for metastasis. Also, stage I patients were excluded because of the very low probability of cancer progression with definitive treatment. Other studies, especially those with low patient numbers, included stage I patients, [5] but larger studies analyzed mainly those with stage II and III disease. [4] When stages II and III were analyzed together, the 5 years survival was 76% for the patients with bone marrow DTC and 59.2% for DTC negative patients. This difference was not statistically significant. These results are contrary to a recent publication from Switzerland, where bone marrow cells were detected with the same type of pan-cytokeratin antibody, with a detection rate similar to ours in patients in stages I-III. The authors found that DTC in the bone marrow was a negative
prognostic factor for survival. [25] The difference with our study was the exclusion of rectal cancer patients and that only about 40% of patients had a left-sided tumor. The cited results were also not supported by others who could not find a prognostic significance of DTC in bone marrow for colorectal cancer patients. [7,20] In all our survival plots, there is a visible trend favoring patients with DTC in bone marrow, but without reaching statistical significance. Therefore, we tried to analyze the incidence of distant metastasis during the 5-year follow up period. In theory, the tumor cells located in the bone marrow could be the source of distant metastasis. There should be a correlation between DTC and metachronous metastasis. Such a situation has been observed in various cancers like breast,[26] prostate [27] and esophageal.[28] Surprisingly, in our study, there was statistically significantly less metachronous distant metastasis in patients with DTC in their bone marrow than in DTC negative patients. The reason for this finding may be the low number of patients however this number is not significantly lower than in the majority of similar studies with the same TNM stages. This finding may indicate that the presence of DTC in some way prevented the progression and metastasis formation. There are theories why DTC do not influence cancer progression, [29] but not about its beneficial effect. Bone marrow is a specific niche with the number of hematopoietic and immunocompetent cells and also stem cells. DTC are present during the generation and maturation of various immunocompetent cells. As was demonstrated, the generation of monocytes from hematopoietic CD34+ stem cells from the bone marrow of colon cancer patients is possible, and these cells act as antigen-presenting cells. They may present tumor-specific antigens to cytotoxic T-lymphocytes. [30] Moreover, it was demonstrated, that after in vivo exposure to blood-derived cell-associated tumor-associated antigens (TAA), BM dendritic cells were capable of inducing systemic protective T cell-mediated anti-tumor immunity upon adoptive transfer. [31] In contrast to BM, responses of lymph nodes to blood-circulating antigen were only weak. There is
evidence that bone marrow-derived dendritic cells differ from peripheral blood-derived dendritic cells, but the importance of this difference is not clear. [32]

Bone marrow also seems to be a preferential site for migration and/or selective retention of memory T-cells and becomes enriched with antigen-specific memory T lymphocytes in response to virus infection or tumor development. [33,34]

Such a mechanism may be the background of the phenomenon observed in our study. DTC surrounded by immune cells may be the source of tumor-related antigens and generate an immune reaction against these antigens. Therefore, DTC may act as a kind of cancer vaccination.

In the previous observations, it has been shown that blood circulating breast cancer cells may settle in the bone marrow and spleen. In these places, antigen-presenting cells may pick-up, process and cross-present tumor-associated antigens to prime naive T lymphocytes. This may lead to generation and maturation of specific effector and also memory T cells. Particular clones of so activated cytotoxic T lymphocytes, upon contact with tumor cells, may exhibit cytotoxic activity or even control dormant cancer foci. [35] It was also proposed that small amounts of persisting tumor-associated antigens produced by dormant tumor cells from the bone marrow may contribute to the maintenance of tumor-specific and long-term memory mediated by appropriate subsets of memory T lymphocytes. [36]

The presence of DTC in the bone marrow is not associated with primary tumor characteristics and seems to diminish metastasis formation in left-sided colorectal cancer. There is a trend for improved overall survival for DCT positive patients.
Acknowledgements

This work was supported by the State Committee of Scientific Research (grant number N N403 089735, AC) and as statutory work at the Jagiellonian University (project number K/ZDS/007895, JB).

Author Contributions

RP, KDT, and AS wrote the manuscript and prepared the tables and figures. RP, KDT, AS, MS, JB, AC critically reviewed the initial version of the manuscript and participated in preparing the final version. JB, MS, AC supervised the whole study and procedures performed to obtain bone marrow status. RP, KDT, JZ, PHB and AS were responsible for data acquisition and statistical analyzes. PHB was responsible for language corrections as a native speaker. AC and JB were responsible for obtaining financial support. All authors reviewed the manuscript and approved the final version.
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### Table 1 Clinicopathological characteristics of the patients

|                                | Number of patients |
|--------------------------------|--------------------|
| **Tumor location**             |                    |
| Left colon                     | 37                 |
| Rectum                         | 54                 |
| **Primary tumor**              |                    |
| T1                             | 2                  |
| T2                             | 16                 |
| T3                             | 60                 |
| T4                             | 13                 |
| **Lymph nodes**                |                    |
| N0                             | 42                 |
| N1                             | 20                 |
| N2                             | 25                 |
| Nx                             | 4                  |
| **Metastases**                 |                    |
| M0                             | 67                 |
| M1                             | 24                 |
| **UICC/AJCC stage**            |                    |
| I                              | 15                 |
| II                             | 26                 |
| III                            | 26                 |
| IV                             | 24                 |
| **Grade**                      |                    |
| 1                              | 26                 |
| 2                              | 45                 |
| 3                              | 13                 |
| not assessed                   | 7                  |
| **Resection margins**          |                    |
| R0                             | 63                 |
| R1                             | 5                  |
| R2                             | 23                 |
Table 2 Number (percentage) of patients with cytokeratins positive cells identified in the bone marrow.

|         | Left Colon 16/37 | Rectum 26/54 | Both locations 42/91 |
|---------|------------------|--------------|----------------------|
| T1-2    | 0/3 (0%)         | 10/15 (66.6%)| 10/18 (55%)          |
| T3      | 13/29 (44.8%)    | 12/31 (38.7%)| 25/60 (41.7%)        |
| T4      | 3/5 (60%)        | 4/8 (50%)    | 7/13 (53.8%)         |
| N-      | 5/15 (33.3%)     | 14/27 (51.9%)| 19/42 (45.2%)        |
| N+      | 10/21 (47.6%)    | 9/24 (37.5%) | 19/45 (42.2%)        |
| Nx      | 1/1 (100%)       | 3/3 (100%)   | 4/4 (100%)           |
| M0      | 11/28 (39.2%)    | 21/39 (53.8%)| 32/67 (47.7%)        |
| M1      | 5/9 (55.5%)      | 5/15 (33.3%) | 10/24 (41.6%)        |
| G1-2    | 12/29 (41.3%)    | 21/42 (50%)  | 33/71 (46.5%)        |
| G3      | 3/6 (50%)        | 0/7 (0%)     | 3/13 (23%)           |
| Gx      | 1/2 (50%)        | 5/5 (100%)   | 6/7 (85.7%)          |
| TNM I   | 0/3 (0%)         | 7/12 (58.3%) | 7/15 (46.6%)         |
| TNM II  | 5/12 (41.7%)     | 7/14 (50%)   | 12/26 (46.2%)        |
| TNM III | 6/13 (46.2%)     | 7/13 (53.8%) | 13/26 (50%)          |
| TNM IV  | 5/9 (55.5%)      | 5/15 (33.3%) | 10/24 (41.6%)        |
| R0      | 10/28 (35.7%)    | 20/35 (57.1%)| 30/63 (47.6%)        |
| R1      | 1/1 (100%)       | 0/4 (0%)     | 1/5 (20%)            |
| R2      | 5/8 (62.5%)      | 6/15 (40%)   | 11/23 (47.8%)        |
Fig 1 Cumulative proportions of survival for disseminated tumor cells positive and disseminated tumor cells negative groups – all tumor stages
Fig 2a Cumulative proportions of survival for patients with stage II and III neoplasm in disseminated tumor cells positive and disseminated tumor cells negative groups
Figure 2b Cumulative proportions of survival for patients with stage II neoplasm in disseminated tumor cells positive and disseminated tumor cells negative groups
Fig 2c Cumulative proportions of survival for patients with stage III neoplasm in disseminated tumor cells positive and disseminated tumor cells negative groups