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Long-term prognostic value of cytokeratin 20 mRNA-positive cells in blood and bone marrow of patients with localized colorectal cancer

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ABSTRACT

Introduction: This study aimed to evaluate whether the presence of circulating and disseminated tumor cells (CTCs/DTCs) in peripheral blood (PB), tumor-draining blood (TDB), and bone marrow (BM) serves as a negative prognostic indicator for long-term cancer-specific survival (CSS) in patients with localized colorectal cancer (CRC). Additionally, associations between CTC/DTC detection and clinical as well as pathological characteristics of the disease were investigated.

Materials and methods: A cohort of 188 patients with localized CRC underwent analysis for the presence of CTCs/DTCs in PB, TDB, and BM. Samples were collected preoperatively, immediately postoperatively, and one month following surgery. Detection was performed via quantitative polymerase chain reaction (qPCR) targeting cytokeratin 20 (CK20) mRNA, an epithelial biomarker, in circulating blood cells and bone marrow specimens.

Results: During a median follow-up of 116.5 months, 81 out of 149 patients (54.7%) with stage I–III CRC succumbed to the disease. Detection of CTCs/DTCs was significantly associated with higher tumor stage and *KRAS* mutation status. The presence of CK20 mRNA-positive CTCs in PB one month after surgery was associated with significantly reduced disease-free survival (DFS), overall survival (OS), and cancer-specific survival (CSS) (log-rank $p = 0.026$, 0.003, and 0.012, respectively). Similarly, patients exhibiting CK20 mRNA-positive DTCs in BM one month postoperatively demonstrated significantly shorter DFS and CSS (log-rank $p = 0.027$ and 0.017, respectively).

Conclusion: Detection of CK20 mRNA-positive CTCs/DTCs in PB and/or BM one month after surgical resection constitutes an independent negative prognostic factor for both long-term DFS and CSS in patients with non-metastatic CRC.

1. Introduction

Colorectal cancer (CRC) is the second leading cause of cancer-related mortality worldwide [1]. Standard treatment modalities include surgical resection, chemotherapy, and for rectal cancer chemoradiotherapy. In the metastatic setting, targeted therapies such as anti-angiogenic agents, epidermal growth factor receptor (EGFR) inhibitors, and immune checkpoint inhibitors are increasingly employed [2].

Despite multimodal curative-intent therapy, 17–24% of patients with localized CRC eventually develop distant metastases [3]. This

Abbreviations: CTC, circulating tumor cell; DTC, disseminated tumor cell; PB, peripheral blood; TDB, tumor-draining blood; BM, bone marrow; CRC, colorectal cancer; CSS, cancer-specific survival; DFS, disease-free survival; OS, overall survival; CEA, carcinoembryonic antigen; hTERT, human telomerase; CK20, cytokeratin 20; EpCAM, epithelial cell adhesion molecule; qPCR, quantitative real-time polymerase chain reaction; EDTA, ethylenediaminetetraacetic acid; CA 19-9, carbohydrate antigen; KRAS, Kirsten rat sarcoma viral oncogene homolog; DNA, deoxyribonucleic acid; mRNA, messenger ribonucleic acid; MSI, microsatellite instability; dNTP, deoxyribonucleotide triphosphate; CFA, colony formation assay; HR, hazard ratio.

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recurrence is partly attributed to understaging and subsequent undertreatment, frequently due to inadequate lymph node sampling [4]. Moreover, staging strategies focused solely on lymph node involvement may not adequately capture the risk of hematogenous dissemination.

Micrometastatic spread, represented by circulating tumor cells (CTCs) in blood and disseminated tumor cells (DTCs) in bone marrow, is believed to be a precursor to overt metastasis [5]. Conventional diagnostic tools lack the sensitivity to reliably detect these minimal residual disease markers. In contrast, molecular methods such as quantitative polymerase chain reaction (qPCR) targeting epithelial markers, including cytokeratin 20 (CK20) gene expression, have shown promise in improving the detection of CTCs and DTCs, thereby identifying patients at elevated risk for recurrence who may benefit from intensified adjuvant treatment [6].

Cytokeratin 20 (CK20) is an intermediate filament protein that is predominantly expressed in gastrointestinal epithelium, particularly in colorectal mucosa and colorectal carcinoma cells, while being largely absent from normal hematopoietic cells. This tissue-restricted expression profile has made CK20 a widely used molecular marker for the detection of circulating tumor cells (CTCs) and disseminated tumor cells (DTCs) by qPCR in patients with colorectal cancer. RT-qPCR-based detection of CK20 mRNA offers high analytical sensitivity, enabling identification of rare epithelial tumor cells in peripheral blood or bone marrow against a background of leukocytes [7].

Previous studies have demonstrated the presence of CTCs in the peripheral blood of CRC patients using various molecular markers and detection platforms. For example, Uen et al. identified a strong association between persistent CTCs postoperatively and shorter disease-free survival (DFS) in 438 stage I–III CRC patients using gene membrane arrays targeting carcinoembryonic antigen (CEA), cytokeratins 19 and 20 (CK19, CK20), and human telomerase reverse transcriptase (hTERT) [8]. Similarly, Iinuma et al. found significantly reduced DFS and overall survival (OS) in Dukes' B and C CRC patients positive for CEA, CK, and CD133 using qPCR [9].

In contrast to blood-based studies, relatively few investigations have addressed the prognostic role of DTCs in bone marrow in CRC. Most available data derive from cohorts of metastatic patients and yield inconsistent conclusions. Some studies (e.g. Hinz et al. [10] and Viehl et al. [11]) found that the presence of DTCs was associated with worse progression-free survival (PFS) and OS. However, other reports, including those by Hinz et al. [12] and Pach et al. [13], did not corroborate these findings.

Studies investigating tumor-draining blood (TDB) are particularly scarce but suggest an adverse prognostic impact of CTCs in this compartment as well [14]. Similarly, the detection of occult tumor cells in peritoneal lavage has been proposed as a relevant prognostic indicator in gastrointestinal malignancies [15].

Two systematic reviews and meta-analyses have confirmed the independent prognostic value of CTCs in CRC [16,17]. Nevertheless, CTC enumeration is not currently incorporated into routine clinical staging or risk stratification. The present study was designed to address this gap by longitudinally assessing the presence of CK20 mRNA-positive CTCs and DTCs using a standardized qPCR method in CRC patients with non-metastatic disease. Specifically, the study aimed to evaluate CTCs in peripheral and tumor-draining blood, and DTCs in bone marrow, at three timepoints, preoperatively, immediately postoperatively, and one month post-surgery. The primary objective was to determine the prognostic significance of CTC/DTC detection for long-term survival outcomes, including DFS and cancer-specific survival (CSS), over a follow-up period exceeding 10 years.

2. Materials and methods

2.1. Patients and sample collection

The study received approval from the Institutional Research Ethics Committee, and written informed consent was obtained from all participants after receiving a detailed explanation of the procedure and its potential risks. It was conducted in accordance with the Declaration of Helsinki and registered at ClinicalTrials.gov (NCT 03337347). Samples of peripheral blood, tumor-draining blood, bone marrow, and tumor tissue were collected from 188 Caucasian patients with colorectal adenocarcinoma at three time points: during anesthesia induction prior to surgery, immediately after tumor resection, and approximately one month postoperatively (median 40 days) (Table 1). The blood samples collected immediately after tumor resection was intended to capture perioperative tumor cell dissemination associated with surgical manipulation and vascular handling. Postoperative sampling at approximately one month after surgery was selected to minimize the impact of perioperative tumor cell release and systemic inflammatory responses, while enabling assessment of persistent circulating and disseminated tumor cells prior to the initiation of adjuvant therapy. This time point also coincided with routine postoperative surgical follow-up, allowing standardized and logistically feasible sample collection.

Peripheral blood was drawn from the cubital vein, while tumor-draining blood was obtained from a major draining vein immediately before vascular clamping. Bone marrow sampling was performed by experienced clinicians via sternal puncture under sterile conditions and was generally well tolerated. No serious procedure-related complications, including major bleeding, infection, sternal injury, or cardiopulmonary events, were observed. Minor, transient local discomfort at the puncture site was occasionally reported and resolved without specific intervention. All blood and bone marrow specimens were drawn into EDTA-containing vacutainer tubes and promptly transported to the laboratory for processing. Total RNA was extracted and reverse transcribed following a previously published protocol [18].

2.2. Determination of CEA and CA 19-9 levels in serum

Serum concentrations of carcinoembryonic antigen (CEA) and carbohydrate antigen 19-9 (CA 19-9) were measured in preoperative peripheral blood samples using an electrochemiluminescence immunoassay (ECLIA; Roche Diagnostics, Mannheim, Germany), following the manufacturer's protocol. CEA levels exceeding 5 μg/L and CA 19-9 levels above 37 kU/L were classified as elevated.

2.3. Detection of KRAS mutations

Mutations in the V-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog (*KRAS*) were analyzed in genomic DNA extracted from tumor tissue using the TheraScreen KRAS Mutation Kit (DxS, Manchester, UK). DNA extraction was performed with the DNeasy Blood and Tissue Kit (Qiagen, Valencia, CA, USA) in accordance with the manufacturer's protocol. The TheraScreen assay detects the seven most prevalent *KRAS* mutations in codons 12 and 13, specifically G12A, G12V, G12C, G12D, G12S, G12R, and G13D.

2.4. Microsatellite instability testing

Microsatellite instability (MSI) in primary tumor and bone marrow samples was assessed using five microsatellite markers recommended by the Bethesda panel: BAT25, BAT26, D2S123, D5S346, and D17S250 [19]. Microsatellite fragment lengths were determined by polymerase chain reaction (PCR) followed by analysis with an Agilent 2100 Bioanalyzer (Agilent, Palo Alto, CA, USA). Samples exhibiting instability at two or more of the tested loci were classified as microsatellite instable (MSI).

2.5. Primers and probe design

To amplify cDNA specific to the epithelial cell marker cytokeratin 20 (CK20), primers and a fluorescent probe were designed using

Table 1
Clinical/pathological characteristics of 188 colorectal cancer patients. (n – number; SE – standard error; OS – overall survival; NR - not reached; CSS – cancer specific survival; KRAS – Kirsten rat sarcoma virus; MSS – microsatellite stable; MSI – microsatellite instable; CEA – carcinoembryonic antigen; CA19-9 – cancer antigen 19-9; T – tumor; N – nodes; M – metastasis).

Clinical stage		1	2	3	4	Total
	(n)	42	59	48	39	188
Sex	**Female/Male**	20/22	28/31	15/33	15/24	78/110
Median age at diagnosis	**Years (quartiles)**	63.5 (59, 73.75)	68 (62.5, 75)	65.5 (54, 73.25)	69 (58.5, 74)	67 (58.75, 74.25)
Overall survival (months)	**5years survival ± SE (%)**	90.5% ± 4.5%	79.7% ± 5.2%	62.5% ± 7%	15.4% ± 5.8%	64.4% ± 3.5%
	median follow-up	171.9	169	112.8	14.6	116.5
	median OS	NR	171.9	112.8	14.6	116.5
Cancer-specific survival (months)	**5years survival ± SE (%)**	95.1% ± 3.4%	90.9% ± 3.9%	64.3% ± 6.9%	16.3% ± 6%	69.3% ± 3.4%
	median follow-up	171.9	169	112.8	14.6	116.5
	median CSS	NR	NR	NR	14.6	NR
***KRAS* status**	**mut/total (%)**	15/37 (40.5%)	17/57 (29.8%)	19/48 (39.6%)	11/29 (37.9%)	62/171 (36.3%)
Microsatellite instability	**MSS/total (%)**	31/36 (86.2%)	48/56 (85.7%)	39/45 (86.7%)	25/27 (92.6%)	143/164 (87.2%)
	MSI/total (%)	5/36 (13.8%)	8/56 (14.3%)	6/45 (13.3%)	2/27 (7.4%)	21/164 (12.8%)
Grading (n)	**1**	10	5	5	6	26
	2	28	43	34	23	128
	3	4	10	5	6	25
Tumor localization (n)	**Colon**	20	42	31	32	125
	Rectum	22	17	17	7	63
Serum CEA	**positive/total (%)**	1/37 (2.7%)	15/52 (28.8%)	6/44 (13.6%)	27/34 (79.4%)	49/167 (29.3%)
Serum CA19–9	**positive/total (%)**	1/37 (2.7%)	3/51 (5.9%)	3/43 (7%)	17/34 (50%)	24/165 (14.5%)
T	**1**	13	0	0	0	13
	2	29	0	5	5	39
	3	0	59	40	18	117
	4	0	0	3	3	6
N	**0**	42	59	0	6	107
	1 + 2	0	0	48	20	68
M	**0**	42	58	48	0	148
	1	0	1	0	39	40
Treatment of colon cancer	**Without anticancer therapy/total (%)**	18/19 (94.7%)	19/37 (51.4%)	2/27 (7.4%)	9/29 (31%)	48/112 (42.9%)
	Only radiotherapy/total (%)	0/19 (0%)	1/37 (2.7%)	0/27 (0%)	0/29 (0%)	1/112 (0.9%)
	Only chemotherapy/total (%)	1/19 (5.3%)	16/37 (43.2%)	25/27 (92.6%)	20/29 (69%)	62/112 (55.4%)
	Concomitant chemoradiotherapy/total (%)	0/19 (0%)	1/37 (2.7%)	0/27 (0%)	0/29 (0%)	1/112 (0.9%)
Treatment of rectal cancer	**Without anticancer therapy/total (%)**	15/19 (78.9%)	6/16 (37.5%)	0/17 (0%)	1/7 (14.3%)	22/59 (37.3%)
	Only radiotherapy/total (%)	2/19 (10.5%)	4/16 (25%)	2/17 (11.8%)	0/7 (0%)	8/59 (13.6%)
	Only chemotherapy/total (%)	2/19 (10.5%)	2/16 (12.5%)	5/17 (29.4%)	6/7 (85.7%)	15/59 (25.4%)
	Concomitant chemoradiotherapy/total (%)	0/19 (0%)	4/16 (25%)	10/17 (58.8%)	0/7 (0%)	14/59 (23.7%)

PrimerPremier3 software based on NCBI reference sequences (accession number NM_019010). The primer pairs were designed to span intron-exon boundaries to avoid amplification of genomic DNA. The sequences used were as follows: forward primer CK20-fw 5′-CGACTTGAACAGGAAATTGCTA-3′, reverse primer CK20-rev 5′-TGCCATCCACTACTTCTTGC-3′, and hydrolysis probe CK20-probe 5′-ACCGCCGCCTTCTGGAAGGA-3′ labeled with BHQ1-HEX, generating a 148 bp amplicon. Primers and probes were synthesized by Generi-Biotech (Hradec Králové, Czech Republic).

2.6. Quantitative polymerase chain reaction

Quantification of CK20 mRNA transcripts was performed using quantitative PCR (qPCR) in a 25 μL reaction volume. Each reaction contained 1 U of HotStart Taq DNA polymerase, 3 mM $MgCl_2$, 1 × PCR buffer (AB Gene, Epsom, UK), 200 μM of each dNTP (Promega, Madison, WI, USA), 400 nM CK20 forward primer, 400 nM CK20 reverse primer, 200 nM CK20 hydrolysis probe, and 100 ng of cDNA. Reactions were run on a Rotor-Gene 3000 thermocycler (Corbett Research, Sydney, Australia) as previously described [20]. The optimized thermal cycling conditions consisted of an initial enzyme activation step at 96 °C for 15 min, followed by 50 amplification cycles of 95 °C for 15 s and 60 °C for 15 s.

2.7. Standardization and normalization of data

Standard curves were generated for each target gene to enable absolute quantification of mRNA copy numbers. PCR amplicons specific to each gene were cloned into the pCR 2.1-TOPO plasmid vector using the TOPO TA Cloning System (Invitrogen, Carlsbad, CA, USA) in accordance with the manufacturer's instructions. Expression of CK20 was normalized to the input RNA quantity used in the reverse transcription reaction to account for intra- and inter-individual variability in reference gene expression. All qPCR experiments were performed in compliance with the Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) guidelines [21].

2.8. Statistical analysis

Statistical analysis was carried out using the R Project for Statistical Computing (www.r-project.org), ver. 4.1.0 and additional R packages: vcd, ver. 1.4-10, epiR, ver. 2.0.41, exactRankTests, ver. 0-8.28, dplyr, ver. 0.4.3 and maxstat, ver. 0-7.23 [22] (Supplementary Methods) (Supplementary Fig. 1). The raw data are accessible at https://doi.org/10.6084/m9.figshare.20156711.v1.

2.9. Verification of qPCR method

To validate the sensitivity of the detection methods, a spiking experiment was conducted using human buffy coat samples spiked with the colorectal adenocarcinoma cell line HT-29 (ATCC HTB-38). Quantitative PCR (qPCR) and colony formation assay (CFA) were performed in parallel. Additionally, the qPCR method was compared with direct microscopic detection of circulating tumor cells (CTCs) using the CytoTrack CT11 system (2C A/S, Copenhagen, Denmark) [23], as detailed in the Supplementary Methods.

3. Results

3.1. Verification of the qPCR method

The qPCR method employed in this study was evaluated against the colony formation assay (CFA) and CytoTrack systems to assess its sensitivity, specificity, and overall feasibility for circulating tumor cell (CTC) detection.

Using CFA, HT-29 colonies were consistently detected across all tested dilutions. Similarly, qPCR analysis showed CK20 mRNA expression levels exceeding the clinically established cut-off values in all dilutions (Supplementary Fig. 2). Both qPCR and CFA reliably detected as few as ten HT-29 cells spiked into buffy coat samples containing 10 million leukocytes. A statistically significant correlation was observed between qPCR and CFA results ($p \leq 0.001$), confirming the consistency of both methods.

Comparison between qPCR and the CytoTrack CT11 system demonstrated superior sensitivity of the qPCR assay in detecting CK20 mRNA-positive CTCs. CTCs were detected in 60% (3/5) of colorectal cancer patients using CytoTrack, whereas qPCR identified CK20-positive CTCs in 80% (4/5) of patients. These findings support the suitability and robustness of the qPCR method for CTC detection in this study.

3.2. Patient characteristics

A total of 256 patients were prospectively enrolled in the study between January 2004 and February 2007. Of these, 68 patients were excluded from the final analyses for the following reasons: 11 patients died within the first month after surgery due to surgical, infectious, or internal complications; 7 patients were diagnosed with a second primary malignancy prior to enrollment; 16 had a prior diagnosis and treatment for colorectal cancer (CRC); 26 had received neoadjuvant chemoradiotherapy before enrollment; and 8 were lost to follow-up. Consequently, 188 patients with histologically confirmed CRC were included in the study (Table 1).

All 149 patients with stage I–III CRC underwent curative (R0) surgical resection, with a median of 14 lymph nodes examined per patient (range: 0–50). All patients were treated and monitored in accordance with standard clinical guidelines [24]. Specifically, among patients with stage I–III colon cancer, adjuvant 5-fluorouracil-based chemotherapy was administered in 5.3%, 48.6%, and 92.6% of stage I, II, and III cases, respectively (Table 1). For rectal cancer patients at stages I–III, 5-fluorouracil-based chemoradiotherapy, including 50.4 Gy of radiation, was administered in 21.1%, 62.5%, and 100% of patients, respectively. In patients with stage IV disease, combination chemotherapy regimens incorporating 5-fluorouracil with either oxaliplatin or irinotecan were used as first-line or progression-directed therapy. Targeted therapies, including bevacizumab or cetuximab, were occasionally employed as second-line treatments in metastatic cases. No patients received immunotherapy. During a median follow-up period of 116.5 months, 81 patients (54.7%) died. CRC-related mortality was observed in 16.6% (3/18), 28.1% (9/32), and 67.7% (21/31) of patients with stage I, II, and III disease, respectively.

3.3. CTC/DTC presence and survival analysis

Disease-free survival (DFS), overall survival (OS), and cancer-specific survival (CSS) analyses were conducted based on the detection of CK20 mRNA-positive circulating tumor cells (CTCs) in peripheral blood, tumor-draining blood, and bone marrow samples collected at three time points: preoperatively, immediately postoperatively, and one month after surgery. The most prognostically significant findings were associated with samples obtained after surgery (Fig. 1).

The presence of CK20 mRNA-positive CTCs in peripheral blood immediately after surgery was significantly associated with shorter DFS (HR = 2.6, $p = 0.024$) and CSS (HR = 2.8, $p = 0.015$). Similarly, CK20 mRNA positivity in peripheral blood one month postoperatively was linked to reduced DFS (HR = 2.4, $p = 0.031$) and CSS (HR = 2.6, $p = 0.016$).

In bone marrow samples collected one month after surgery, the presence of CK20 mRNA-positive disseminated tumor cells (DTCs) was also associated with poorer outcomes, showing shorter DFS (HR = 2.2, $p = 0.031$) and CSS (HR = 2.4, $p = 0.021$).

In contrast, the preoperative detection of CK20 mRNA-positive CTCs in peripheral blood, tumor-draining blood, or bone marrow did not demonstrate a statistically significant association with survival outcomes. Additionally, no significant differences in survival were found when analyzing the presence of CTCs in tumor-draining blood in colon and rectal cancer patients.

Multivariable Cox models adjusted for age at diagnosis, sex and tumor stage confirmed the negative prognostic impact of CK20 mRNA-positive CTCs one month postoperatively on DFS (HR = 3.1, $p = 0.009$) and CSS (HR = 3.5, $p = 0.003$) in peripheral blood, and on DFS (HR = 2.3, $p = 0.032$) and CSS (HR = 2.3, $p = 0.030$) in bone marrow. Adjusted models for OS could not be applied due to the non-proportional hazards related to age.

Furthermore, a multivariate Cox regression model with stepwise variable selection identified age, gender, tumor location, and CK20 mRNA expression in peripheral blood one month after surgery as independent negative prognostic factors for CSS.

In tumor location–specific subgroup analyses, the associations between the presence of CTCs or DTCs and survival outcomes were more pronounced and statistically significant in patients with colon cancer, whereas no significant prognostic impact was observed in patients with rectal cancer. Among patients with colon cancer, the presence of CK20 mRNA–positive CTCs in peripheral blood collected one month after surgery was significantly associated with shorter DFS (HR = 4.3, p = 0.006) and CSS (HR = 5.7, p = 0.001) (Supplementary Fig. 3). Similarly, in bone marrow samples obtained one month postoperatively, the presence of CK20 mRNA–positive DTCs was associated with inferior outcomes, including reduced DFS (HR = 3.7, p = 0.009) and CSS (HR = 4.6, p = 0.004) (Supplementary Fig. 4).

3.4. Tumor MSI and KRAS gene mutation status

Formalin-fixed, paraffin-embedded tumor tissue samples were analyzed for microsatellite instability (MSI) and *KRAS* gene mutations in 164 and 171 colorectal cancer (CRC) patients, respectively. MSI was detected in 12.8% of cases, while mutations in codons 12 and 13 of the *KRAS* gene were identified in 36.3% of patients (Table 1). Neither MSI nor KRAS mutation status was significantly associated with survival outcomes in this cohort (Fig. 2). Notably, CK20 mRNA-positive cells were more frequently detected in peripheral blood, tumor-draining blood, and bone marrow samples from patients with wild-type *KRAS* tumors (Table 2).

3.5. CTC/DTC presence and disease characteristics

Classical prognostic factors, including depth of invasion, lymph node status, tumor grade, and serum levels of CEA and CA 19-9, were assessed

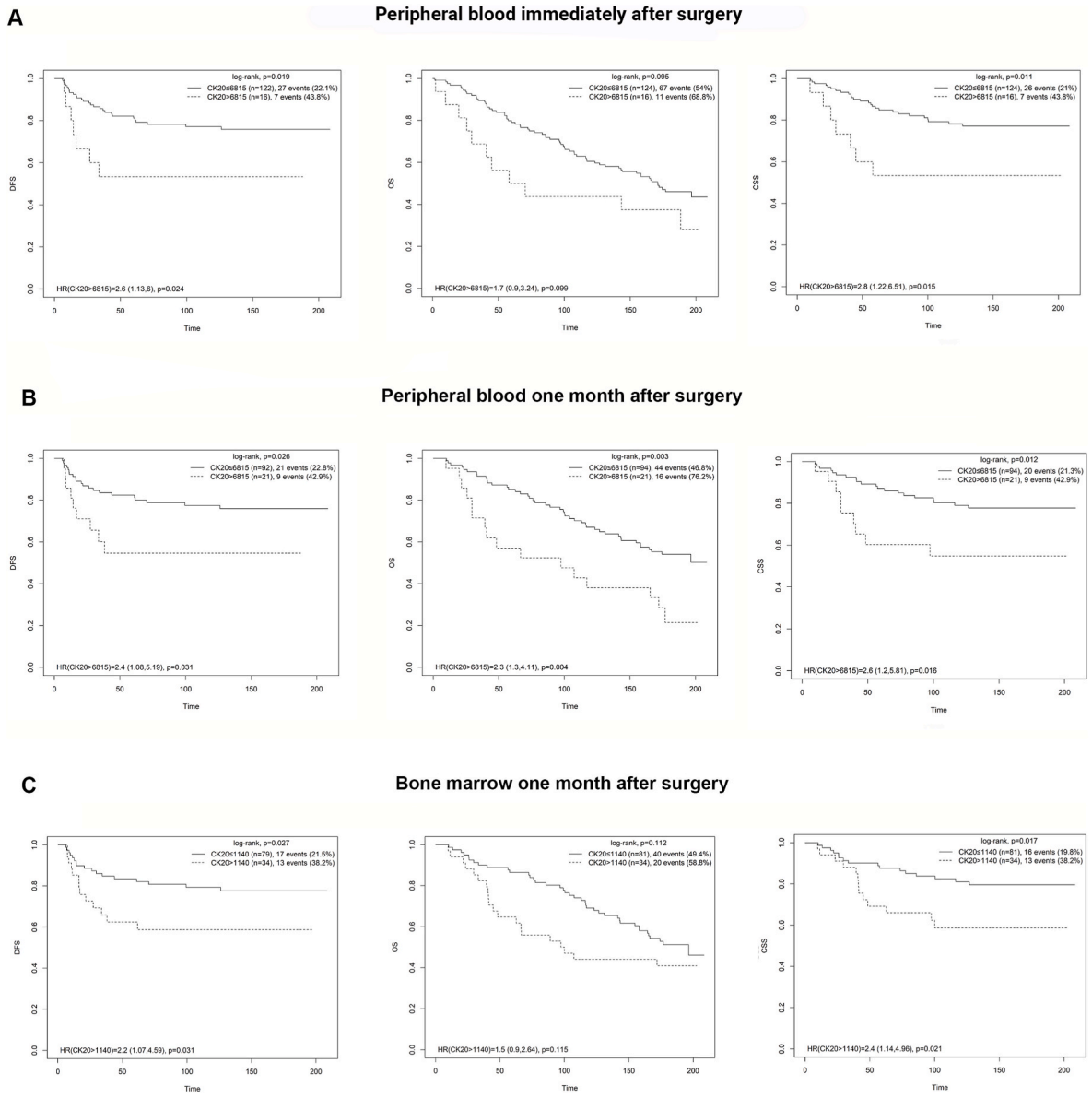

Fig. 1. Kaplan–Meier survival curves illustrating disease-free survival (DFS), overall survival (OS), and cancer-specific survival (CSS) in stage I–III colorectal cancer patients, stratified by the presence or absence of CK20 mRNA-positive circulating and disseminated tumor cells (CTCs/DTCs) detected via qPCR in peripheral blood collected immediately after surgery, and in peripheral blood and bone marrow samples collected one month postoperatively.

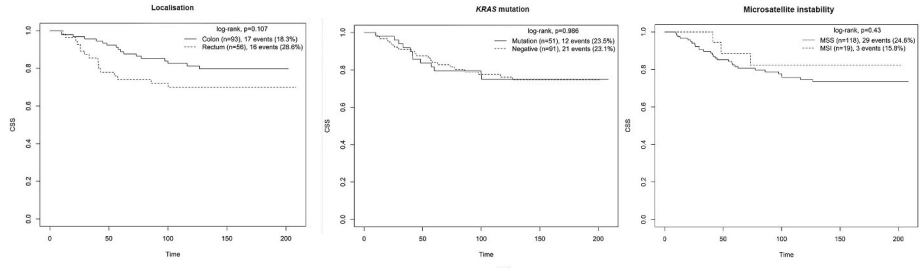

Fig. 2. Kaplan–Meier curves illustrating cancer-specific survival in stage I–III colorectal cancer patients, stratified by KRAS mutation status, tumor localization, and microsatellite instability (MSI) status.

for their association with the presence of circulating and disseminated tumor cells (CTCs/DTCs). Elevated levels of CK20 mRNA-positive cells were observed in postoperative peripheral blood samples from patients with more advanced disease. Notably, CTCs were significantly more frequent in patients with lymph node metastases ($p = 0.045$) (Table 2). No other clinicopathological parameters showed a significant correlation with CTC levels.

Table 2
Relationship between clinical/pathological features of colorectal cancer patients and circulating/disseminated tumor cells' presence in peripheral blood, tumor-draining blood and bone marrow. (CK20 –cytokeratin 20; mRNA – messenger ribonucleic acid; CTCs – circulating tumor cells; DTCs – disseminated tumor cells; PB – peripheral blood; TDB – tumor draining blood; BM – bone marrow; T – tumor; N – nodes; CEA – carcinoembryonic antigen; CA19-9 – cancer antigen 19-9; KRAS – Kirsten rat sarcoma virus; MSS – microsatellite stable; MSI – microsatellite instable). *$p < 0.05$ (Pearson's chi-square test or Fisher's exact test) #$p < 0.05$ (Pearson's chi-square test or Fisher's exact test), Bonferroni corrected.

		PB before surgery	PB immediatelly after surgery	PB 1 month after surgery	TDB before surgery	TDB immediatelly after surgery	BM before surgery	BM 1 month after surgery
Cut-off values (mRNA copies/μg RNA)		6815	6815	6815	3020	3020	1140	1140
Clinical stage			*#	*#	*			
	1	7/42 (16.7%)	2/35 (5.7%)	11/29 (37.9%)	15/34 (44.1%)	10/30 (33.3%)	7/42 (16.7%)	12/29 (41.4%)
	2	11/59 (18.6%)	3/57 (5.3%)	2/45 (4.4%)	18/48 (37.5%)	16/47 (34%)	12/59 (20.3%)	7/45 (15.6%)
	3	6/48 (12.5%)	11/48 (22.9%)	8/41 (19.5%)	24/46 (52.2%)	16/42 (38.1%)	10/48 (20.8%)	15/41 (36.6%)
	4	9/39 (23.1%)	7/26 (26.9%)	3/19 (15.8%)	16/21 (76.2%)	10/22 (45.5%)	8/39 (20.5%)	7/20 (35%)
Age median (q25-q75)	Negative (≤cut-off)	67 (59-75)	67 (59-75)	67 (59-73.75)	69 (59.75-74.25)	66 (57-75)	67 (58-75)	67 (59-74)
	Positive (>cut-off)	68 (56-72)	68 (53.5-72)	67.5 (54.25-73.5)	67 (57-75)	68 (61.25-75)	68 (61-73)	65 (54-72)
T				*				
	1	2/13 (15.4%)	0/9 (0%)	0/6 (0%)	5/9 (55.6%)	3/8 (37.5%)	2/13 (15.4%)	1/6 (16.7%)
	2	6/39 (15.4%)	3/36 (8.3%)	12/32 (37.5%)	14/35 (40%)	9/31 (29%)	8/39 (20.5%)	15/32 (46.9%)
	3	21/117 (17.9%)	16/111 (14.4%)	12/91 (13.2%)	50/100 (50%)	35/96 (36.5%)	26/117 (22.2%)	22/91 (24.2%)
	4	1/6 (16.7%)	3/6 (50%)	0/3 (0%)	4/5 (80%)	4/5 (80%)	1/6 (16.7%)	2/4 (50%)
N			*#					
	0	18/107 (16.8%)	7/98 (7.1%)	13/79 (16.5%)	37/86 (43%)	28/81 (34.6%)	21/107 (19.6%)	20/79 (25.3%)
	1 + 2	12/68 (17.6%)	15/64 (23.4%)	11/53 (20.8%)	36/63 (57.1%)	23/59 (39%)	16/68 (23.5%)	20/54 (37%)
Grading				*				
	1	16/26 (61.5%)	12/23 (52.2%)	13/18 (72.2%)	9/23 (39.1%)	9/22 (40.9%)	3/26 (11.5%)	3/18 (16.7%)
	2	55/128 (43%)	57/118 (48.3%)	35/98 (35.7%)	50/106 (47.2%)	40/99 (40.4%)	15/128 (11.7%)	11/96 (11.5%)
	3	13/25 (52%)	8/23 (34.8%)	9/19 (47.4%)	6/21 (28.6%)	9/19 (47.4%)	4/25 (16%)	2/18 (11.1%)
Serum CEA								
	Negative	20/118 (16.9%)	13/109 (11.9%)	19/90 (21.1%)	47/104 (45.2%)	34/96 (35.4%)	23/118 (19.5%)	32/90 (35.6%)
	Positive	12/49 (24.5%)	9/39 (23.1%)	4/27 (14.8%)	20/31 (64.5%)	14/30 (46.7%)	11/49 (22.4%)	5/28 (17.9%)
Serum CA 19-9			*					
	Negative	29/141 (20.6%)	16/129 (12.4%)	20/103 (19.4%)	60/122 (49.2%)	43/113 (38.1%)	32/141 (22.7%)	33/104 (31.7%)
	Positive	3/24 (12.5%)	6/17 (35.3%)	3/12 (25%)	6/12 (50%)	5/12 (41.7%)	2/24 (8.3%)	4/12 (33.3%)
KRAS		*#						
	Wild-type	25/109 (22.9%)	18/104 (17.3%)	17/80 (21.2%)	49/94 (52.1%)	38/88 (43.2%)	27/109 (24.8%)	24/80 (30%)
	Mutation	3/62 (4.8%)	5/58 (8.6%)	7/50 (14%)	23/53 (43.4%)	14/52 (26.9%)	8/62 (12.9%)	16/51 (31.4%)
Microsatellite instability	MSS	15/100 (15%)	13/94 (13.8%)	14/68 (20.6%)	42/84 (50%)	27/77 (35.1%)	18/100 (18%)	19/69 (27.5%)
	MSI	11/64 (17.2%)	9/61 (14.8%)	8/54 (14.8%)	28/55 (50.9%)	21/54 (38.9%)	17/64 (26.6%)	19/54 (35.2%)
Tumor localization								
	Colon	21/125 (16.8%)	14/107 (13.1%)	15/88 (17%)	51/98 (52%)	36/96 (37.5%)	21/125 (16.8%)	26/89 (29.2%)
	Rectum	12/63 (19%)	9/59 (15.3%)	9/46 (19.6%)	22/51 (43.1%)	16/45 (35.6%)	16/63 (25.4%)	15/46 (32.6%)

4. Discussion

This long-term follow-up study evaluated the prognostic significance of circulating (CTCs) and disseminated tumor cells (DTCs) in non-metastatic colorectal cancer (CRC), with a particular focus on CK20 mRNA detection using a sensitive qPCR-based approach. While previous studies have consistently demonstrated the prognostic value of CTCs in CRC [16,17], the role of DTCs, especially in bone marrow, remains unclear, with conflicting results reported in the literature [10,11,13].

Our data show that DTCs were detectable in up to 30% of early-stage CRC patients and were significantly associated with reduced disease-free survival (DFS) and cancer-specific survival (CSS), supporting the

concept that bone marrow may serve as a permissive microenvironment for cancer cell survival and dissemination [25]. These findings align with the study by Viehl et al., which reported worse DFS and OS in DTC-positive patients after a 6.2-year median follow-up [11].

Importantly, the timing of sample collection proved critical for assessing the prognostic value of CTCs/DTCs. Only a limited number of studies have analyzed serial samples due to the logistical challenges of repeated sampling. Dalum et al. found preoperative CTCs correlated with recurrence-free survival (RFS), while postoperative CTCs lacked significance [26]. However, several other studies have shown prognostic relevance of postoperative CTC detection in non-metastatic CRC [8,9]. A meta-analysis by Yang et al. reported no consistent impact of timing on prognostic value, highlighting the need for further investigation [27].

In our study, the detection of CK20 mRNA–positive circulating tumor cells immediately after surgery was associated with shorter disease-free and cancer-specific survival. This observation should not be interpreted as a rapid biological change occurring within minutes after tumor resection, but rather as a reflection of inter-individual differences in tumor biology. Tumors with greater invasive capacity and vascular dissemination potential may release detectable tumor cells during surgical manipulation, thereby identifying patients with aggressive disease biology and occult micrometastatic burden that are not fully captured by conventional pathological staging parameters, including lymph node status and resection margin assessment.

In our cohort, the detection of CK20 mRNA-positive CTCs in peripheral blood and CK20 mRNA-positive DTCs in bone marrow one month after surgery was associated with significantly shorter DFS, OS, and CSS. Given that non-cancer-related deaths may influence OS over long-term follow-up, CSS was deemed a more accurate reflection of cancer-specific outcomes. We found a borderline impact of CTCs/DTCs on OS and confirmed a significant impact of CTCs/DTDs on CSS in our long-term follow-up study.

Interestingly, unlike previous studies [14], we observed no survival differences associated with CTCs in tumor-draining blood, despite analyzing one of the largest cohorts to date (157 patients with stage I–III CRC) and the longest median follow-up. This may reflect the hepatic portal system's efficient clearance of tumor cells and its “first-pass” filtering effect [28,29].

Subgroup analysis indicated that CTCs may lack clear prognostic value in rectal cancer because disease outcome is more strongly determined by local disease control than by hematogenous dissemination. In rectal cancer, recurrence is frequently driven by local or locoregional relapse related to surgical factors, rather than by early distant spread. As a result, CTCs detected in peripheral blood primarily reflecting hematogenous dissemination may not adequately capture the dominant mechanisms of disease progression in this setting. In addition, the rectum has a mixed venous drainage into both the portal and systemic circulation, which may weaken the association between CTC detection in peripheral blood and subsequent metastatic relapse. Biological heterogeneity of rectal tumors, including epithelial–mesenchymal plasticity and reduced expression of epithelial markers such as CK20, may further limit the sensitivity and prognostic relevance of CTC detection methods. Together, these factors likely explain the limited prognostic significance of CTCs in non-metastatic rectal cancer [30].

Regarding clinicopathological correlations, previous reports have shown mixed results [9,26,29,31]. In our analysis, CK20 mRNA-positive CTCs/DTCs were more frequent in patients with advanced disease and KRAS wild-type tumors, particularly in peripheral blood samples. This may suggest a selective clearance or hepatic tropism of KRAS-mutant tumor cells, consistent with the higher incidence of liver metastases in these patients [32]. No other clinical or molecular features (e.g., MSI, serum CEA/CA19-9, tumor location) correlated significantly with CTC/DTC presence.

Based on multivariate analyses and previously published evidence [9,12,31], we propose that postoperative detection of CTCs/DTCs is an independent prognostic factor in non-metastatic CRC. From a clinical perspective, peripheral blood sampling is clearly preferable, as bone marrow sampling is more invasive and ethically contentious, particularly when it does not provide additional prognostic value.

However, our study was limited by its single-center design and the inclusion of only Caucasian patients, which may restrict the generalizability of the findings to colorectal cancer populations of other ethnicities and geographic regions.

Emerging CTC detection platforms, such as CellSearch® (Menarini), use immunomagnetic enrichment and epithelial markers for direct visualization and enumeration of CTCs. While this method offers phenotypic characterization, its reliance on EpCAM presents limitations due to potential downregulation during epithelial-mesenchymal transition (EMT), which may lead to false-negative results [33]. Furthermore, clinical trials using anti-EpCAM antibodies in metastatic CRC failed to demonstrate benefit, questioning its suitability as a universal CTC marker.

By contrast, qPCR-based detection of CK20 mRNA offers higher sensitivity and requires only standard molecular biology equipment. However, this approach carries a risk of false-positive results, arising from illegitimate transcription of epithelial markers in non-epithelial cells or from contamination with epithelial cells during blood sampling. Despite the high analytical sensitivity of qPCR, a residual risk of false-negative results remains, primarily due to absent, heterogeneous or downregulated CK20 expression in tumor cells and to mRNA degradation during the pre-analytical phase [7]. Recently developed approaches may enhance CTC detection specificity and overcome some of these limitations [34].

In summary, this study featuring one of the largest and longest-followed cohorts of non-metastatic CRC patients, demonstrates that detection of CK20 mRNA-positive CTCs and DTCs using qPCR is significantly associated with DFS and CSS. Our findings support the prognostic relevance of CTCs/DTCs, particularly when detected postoperatively, and underscore the clinical practicality of peripheral blood analysis over bone marrow sampling. Although CTCs in tumor-draining blood did not correlate with survival in this study, this remains a novel and underexplored area with potential mechanistic implications.

5. Conclusion

In conclusion, this study confirms that samples collected approximately one month after surgery hold prognostic value for identifying colorectal cancer patients at increased risk of recurrence, consistent with previous findings. These high-risk individuals may benefit from adjuvant therapy aimed at eliminating residual CTCs/DTCs. Nonetheless, the optimal timing for sample collection remains to be fully established.

CRediT authorship contribution statement

Josef Srovnal: Writing – review & editing, Writing – original draft, Validation, Project administration, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Pavel Skalicky:** Writing – review & editing, Writing – original draft, Resources, Methodology, Investigation, Data curation. **Andrea Prokopova:** Writing – review & editing, Methodology, Investigation, Data curation. **Jana Vrbkova:** Writing – review & editing, Visualization, Software, Data curation. **Monika Vidlarova:** Writing – review & editing, Validation, Methodology, Investigation, Data curation. **Miloslav Duda:** Writing – review & editing, Supervision, Resources, Project administration, Funding acquisition, Conceptualization. **Kamil Vyslouzil:** Writing – review & editing, Resources, Methodology, Investigation, Data curation, Conceptualization. **Marian Hajduch:** Writing – review & editing, Writing – original draft, Supervision, Resources, Project administration, Funding acquisition, Formal analysis, Data curation, Conceptualization.

Role of the funding source

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Declaration of competing interest

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Appendix A. Supplementary data

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