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Advances in Brief

Comparative Analysis of Bone Marrow and Venous Blood Isolates from Gastrointestinal Cancer Patients for the Detection of Disseminated Tumor Cells Using Reverse Transcription PCR[1]

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Abstract

We investigated 141 bone marrow and 104 venous blood isolates from gastrointestinal cancer patients with a cytokeratin (CK) 20-specific nested reverse transcription PCR for the detection of disseminated tumor cells at time of primary tumor resection. In colorectal cancer patients, 20 of 65 (31%) bone marrow and 9 of 52 (17%) venous blood isolates yielded a CK 20 mRNA-positive result in a stage-dependent manner. The detection rates for gastric cancer patients were 11 of 49 (22%) and 5 of 30 (17%) for bone marrow and venous blood, respectively. In pancreatic cancer patients, positive signals were found in advanced tumor stage. A duplex PCR system improved the feasibility of the test. After analyzing 70 sets of bone marrow and venous blood isolates from colorectal, gastric, and pancreatic cancer patients, we observed a higher detection rate in bone marrow isolates. Survival of patients with CK 20 mRNA-positive findings was significantly shorter than that of negatively tested patients.

Introduction

The development of metastasis in cancer is one of the main problems after primary tumor resection. The identification of metastases is only possible in the follow-up investigation when there is already a solid tumor mass. Subclinical tumor cell dissemination can be detected by immunocytological staining of cells (1, 2) or by other molecular biological methods, like PCR. The molecular biological approach addresses differences in DNA structure, like point mutation (3) and gene rearrangement (4) or expression of tissue-specific mRNAs (5). We used a RT-PCR[3] specific for the intermediate filament CK 20 (6, 7) as a sensitive and specific marker for early tumor cell dissemination of gastrointestinal carcinoma. Some patient specimens were screened with a modified (duplex) version of the previously described CK 20-specific RT-PCR (8), which was more convenient. Overall, an extended panel of bone marrow (n = 141) and venous blood (n = 104) isolates from patients suffering from colorectal, gastric, and pancreatic carcinoma and undergoing primary tumor resection was investigated. The following questions were answered: (*a*) Is the CK 20 duplex RT-PCR approach able to replace the separate CK 20 RT-PCR and GAPDH-PCR step? (*b*) Is the CK 20 RT-PCR able to elucidate whether gastric carcinoma patients are already afflicted with disseminated disease? (*c*) Is the CK 20 RT-PCR also a powerful detection method in venous blood? (*d*) If so, is the CK 20 mRNA testing of the bone marrow replaceable by venous blood investigation? (*e*) Does CK 20 mRNA detection in bone marrow and venous blood have a prognostic value referring to the survival of the patients?

Materials and Methods

Citrated bone marrow and venous blood isolates (20 ml each) were obtained from gastrointestinal cancer patients and patients without apparent carcinoma as control group. Samples were taken from patients prior to tumor resection. Patients suffering from tumor recurrence or metachronous metastasis were excluded. Table 1 summarizes those samples that showed sufficient yield of RNA for testing.

All patients were informed about the study and gave written consent. Sampling procedure and cell isolation have been described previously (2).

Isolation of Total RNA. Nucleated cells were isolated on Ficoll-Isopaque (Pharmacia, Freiburg, Germany) and then lysed in TRIzol LS Reagent (Life Technologies, Eggenstein, Germany). RNA was isolated according to the single-step isolation method (9). To optimize the yield of RNA, 5 μg of glycogen (Boehringer Mannheim, Mannheim, Germany) were added to the aqueous phase before precipitation with isopropanol. Each RNA preparation was dissolved in 20 μl RNase free water. The amount and purity of RNA from each sample was determined by absorption measurement at 260 and 280 nm (Gene Quant II, Pharmacia).

RT-PCR. Total RNA (2.0 μg) in a volume of 10 μl was denatured for 10 min at 70°C and quickly chilled on ice. The cDNA was synthesized in a total volume of 20 μl containing 5× first-strand buffer, 2 mM DTT, 200 units of SuperScript II (all from Life Technologies), 4 units of RNase inhibitor, 2.5 μM random hexamer, 0.5 mM of each deoxynucleotide triphosphate mixture (all from Perkin-Elmer Corp., Weiterstadt, Germany). Incubation for 10 min at 24°C and 60 min at 42°C was followed by an inactivation step for 5 min at 95°C. Primers were synthesized by MWG-Biotech, Ebersberg, Germany. CK 20-A sense, 5'-GCGTTTATGGGGGTGCTGGAG; CK 20-B antisense, 5'-AAGGCTCTGGGAGGTGCGTCTC; CK 20-C sense, 5'-CGGCGGGGACCTGTTTGT; CK 20-D antisense, 5'-CAGTGTTGCCCAGATGCTTGTG; GAPDH sense, 5'-CCAGCCGAGCCACATCGCTC; and GAPDH antisense, 5'-ATGAGCCCCAGCCTTCTCCAT. A volume of 30 μl of a PCR mixture containing 10 × tricine buffer III (10), 200 μM deoxynucleotide triphosphate mixture, CK 20-A and CK 20-B (0.4 μM each), and 1 unit of Taq DNA polymerase (Life Technologies) was added to the cDNA preparation (20 μl). The cycling protocol for PCR is listed in Table 2. Nested duplex PCR was performed essentially as described above for the external PCR. One μl of the first PCR reaction was formulated with 0.4 μM CK 20-C and CK 20-D and 0.2 μM of each GAPDH primer in a final volume of 50 μl. The nested CK 20 PCR yielded a 485-bp product, distinct from the GAPDH product, which had a length of 359 bp. This refinement of our previously published work (8) was verified, as was a positive control (50 pg of total RNA from the pancreatic carcinoma cell line A818–4 in 2 μg of venous blood RNA from a healthy donor). In the reagent mixture control RNA was replaced by RNase-free water.

Evaluation Criteria. Samples were tested at least twice. Two identical results completed testing. A few samples that gave contrasting results were tested up to four times. If the test was, for example, negative three times and positive once, the sample was declared negative.

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[3] The abbreviations used are: RT-PCR, reverse transcription PCR; CK, cytokeratin; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; UICC, Union Internationale contre le Cancer.

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Table 1 *Total number of patient RNA samples*

Samples	Bone marrow	Paired samples[a]	Venous blood
Colorectal tumor	65	39	52
Gastric tumor	49	18	30
Pancreatic tumor	27	13	22
Controls[b]	22	12	58
Total	163	82	162

Rows show grouping of samples according to the tumor location and control samples; columns show total number of RNA samples from bone marrow and venous blood aspirates.
[a] Means set of bone marrow and venous blood RNA from the same patient.
[b] Patients without apparent carcinoma and healthy donors.

Table 2 *Cycling protocol for CK 20 PCR*

Step	Denaturation time and temperature	Annealing and extension time and temperature		Cycle no.
1	40 s/94°C	2 min/70°C		1
2	40 s/94°C	1 min 55 s/69°C		1
3	40 s/94°C	1 min 50 s/68°C		1
4	40 s/94°C	1 min 45 s/67°C		1
5	40 s/94°C	1 min 40 s/66°C		1
6	40 s/94°C	1 min 35 s/65°C		1
7	40 s/94°C	1 min 30 s/64°C		1
8	40 s/94°C	1 min 25 s/63°C		1
9	40 s/94°C	1 min 20 s/62°C		1
		Annealing time and temperature	Extension time and temperature	
10–30	40 s/94°C		1 min 30 s/72°C	21
31		1 min/61°C	15 min/72°C	

Results

Overall, with the CK 20-specific nested RT-PCR, 141 bone marrow and 104 venous blood samples from gastrointestinal cancer patients undergoing primary tumor resection were screened. Patients with tumor recurrence were excluded from the study.

Nested Duplex CK 20 RT-PCR. We improved the PCR protocol to a more rapid approach toward clinical application. For monitoring of the cDNA synthesis, the GAPDH PCR was not performed as a separate step, but was done simultaneously with the nested CK 20 amplification. Forty arbitrarily chosen bone marrow and blood isolates of gastrointestinal cancer patients were investigated with the established CK 20 RT-PCR (8) and simultaneously with the duplex approach. Both procedures gave identical information of CK 20 positivity. No decrease in sensitivity or specificity using the duplex approach was obtained. The positive control of 50 pg of total RNA from A818-4 cells spiked in a total of 2 μg of venous blood RNA reflects approximately 5–10 tumor cells in $2–4 \times 10^6$ lymphocytes and monocytes isolated through Ficoll preparation. A representative collection of four sets of bone marrow and blood samples from colorectal cancer patients and four sets of isolates from gastric cancer patients investigated with the established CK 20 RT-PCR and GAPDH RT-PCR in parallel to the refined duplex CK 20 RT-PCR is illustrated in Fig. 1.

Colorectal Cancer Patients. Staging of the patients was performed according to UICC classification (11). Twenty of 65 (31%) patients exhibited a CK 20-specific signal in bone marrow RNA. Comparison of the RT-PCR results are shown in Fig. 2*A* (*filed columns*): 1 of 8 patients with stage I (T1,2 N0 M0) tumor, 3 of 22 (14%) with stage II (T3,4 N0 M0), 4 of 12 (25%) with stage III (TX N1,2,3 M0), and 12 of 23 (52%) patients with stage IV (TX NX M1) malignant disease showed CK 20-positive results in bone marrow. Investigation of the venous blood samples yielded an overall positivity of 17% (9 of 52). Results are given in Fig. 2*A* (*open columns*): 0 of 8 with a stage I tumor, 1 of 17 (6%) with stage II, 1 of 9 (11%) with stage III, and 7 of 18 (39%) with stage IV malignant disease. Table 3 comprises the data obtained from sets of samples (n = 39), in which bone marrow and venous blood were tested in parallel. In stage I, 5 of 6 were completely negative, and one set was positive in bone marrow but negative in venous blood. In stage II, 12 of 13 pairs gave equivalent results; *i.e.*, 1 positive set and 11 negative sets. Again, one set tested positive in bone marrow and negative in blood. Stage III (n = 4) has given only negative results thus far. The stage IV group gave 14 of 16 equivalent results in the compartments, with 6 positive and 8 negative pairs. The 2 of 16 divergent sets exhibited a positive signal in bone marrow but tested negative in blood.

Overall, 35 of 39 (90%) of the tested sets exhibited identical results, of these, 7 were positive and 28 were negative. All four pairs with divergent results were positive in bone marrow and negative in venous blood.

Gastric Cancer Patients. Eleven of 49 (22%) patients exhibited a CK 20-specific signal in bone marrow RNA. Comparison of the RT-PCR results is shown in Fig. 2*B* (*filled columns*): 0 of 12 patients with a stage I (T1 N0,1 M0; T2 N0 M0) tumor, 1 of 5 (20%) with stage II (T1 N2 M0; T2 N1 M0; T3 N0 M0), 3 of 14 (21%) with stage III (T2 N2 M0; T3 N1,2 M0; T4 N0,1,2 M0), and 7 of 18 (39%) with stage IV (T4 N2 M0; TX NX M1) carcinoma revealed a CK 20-specific signal in our assay. Investigation of venous blood samples yielded an overall positivity of 17% (5 of 30) and is demonstrated in Fig. 2*B* (*open columns*): 2 of 9 with a stage I tumor, 1 of 5 with stage II, 0 of 5 with stage III, and 2 of 11 with stage IV. Table 3 contains the data obtained from sets of samples (n = 18), in which bone marrow and venous blood were tested in parallel. Specimens received from patients in stage I (n = 4) gave only negative results. In stage II (n = 2), one set was negative, and the other set exhibited a positive signal in bone marrow but was negative in blood. In stage III (n = 4), only negative results were observed. The stage IV group revealed 5 of 8 equivalent results in both compartments, with one positive pair and four negative pairs. Three of 8 divergent sets exhibited a positive signal in bone marrow but tested negative in blood. Overall, 14 of 18

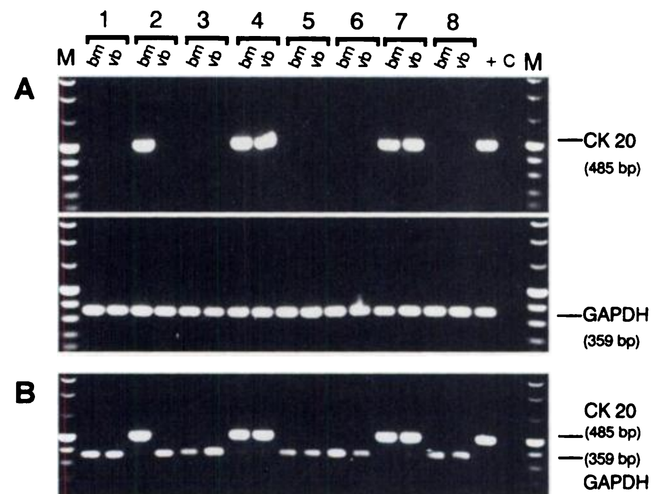

Fig. 1. CK 20-specific nested RT-PCR and GAPDH RT-PCR in comparison with the duplex approach. Samples were separated by agarose-gel electrophoresis and stained with ethidium bromide. Eight arbitrarily chosen sets of bone marrow (*bm*) and venous blood (*vb*) isolates from colorectal (1–4) and gastric cancer patients (5–8) were demonstrated. +, positive control (50 pg total RNA from pancreatic carcinoma cell line A818–4 in 2 μg of venous blood from a healthy donor); *C*, reagent mixture control; *M*, molecular weight marker. *A*, upper band, CK 20 nested RT-PCR (485 bp); lower band, GAPDH RT-PCR (359 bp). *B*, duplex approach with amplification of the CK 20 cDNA product; only a weak GAPDH cDNA product was visible. CK 20-negative isolates yielded in a strong GAPDH signal. Patient 2 was CK 20 positive in bone marrow but negative in venous blood and patients 4 and 7 were positive in bone marrow and venous blood; the other patients tested negative in both compartments.

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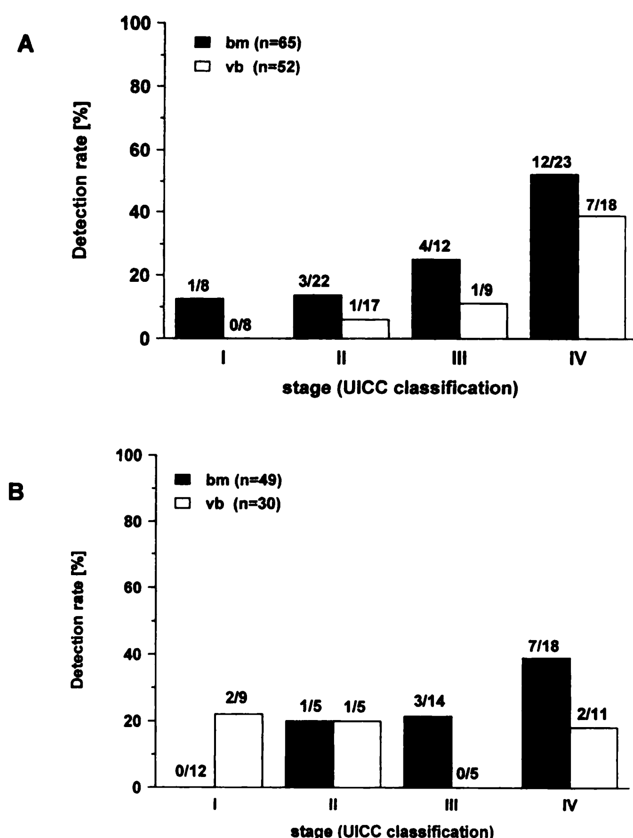

Fig. 2. Comparison of CK 20 RT-PCR positive results in bone marrow (*bm*) and venous blood (*vb*) with the stage of disease (UICC classification). The number of positive cases and the total number of cases are shown by the numbers before and after the slash, respectively, above the columns. *A*, colorectal cancer patients; *B*, gastric cancer patients.

of the tested sets exhibited identical results; of these, 1 pair was positive, and 13 pairs were negative. The four pairs with divergent result were always positive in bone marrow and negative in venous blood.

Pancreatic Cancer Patients. In this group, 5 of 27 (19%) bone marrow samples were positive in our test. All 5 positive samples resulted from the stage IV (TX NX M1) tumor group, which included 17 samples. Investigation of the blood samples ($n = 22$) revealed only two positive specimens, belonging to the advanced tumor stages III and IV. Table 3 comprises the data obtained from the sets of samples ($n = 13$). Overall, 12 of 13 sets were found to be negative; 1 set was positive in bone marrow and negative in blood.

Controls. In bone marrow ($n = 22$) from patients without apparent carcinoma, we observed CK 20-positive results in two samples. One patient suffered from familial adenomatous polyposis, and the other suffered from an extended liver adenoma type I. Investigation of the venous blood control group ($n = 58$) gave positive results in two patients: one patient with liver adenoma type I, who was tested positive in bone marrow as well, and one patient with chronic pancreatitis.

Prognostic Value of the CK 20 RT-PCR for Colorectal and Gastric Cancer Patients. The follow-up studies concentrating on the survival (Kaplan-Meier calculation) of the colorectal and gastric cancer patients tested in both compartments are shown in Fig. 3. In the colorectal cancer group (Fig. 3*A*), patients with CK 20-positive results in both compartments or positive results in the bone marrow but negative results in blood ($n = 10$) had a significantly shorter survival ($P > 0.0001$) than patients tested negative in both compartments ($n = 27$). Two patients, one with positive testing, the other with negative testing in both compartments, were excluded from the Kaplan-Meier calculation. Both patients died due to surgical complications. In the gastric cancer group (Fig. 3*B*), patients with CK 20-positive results in both compartments or positive results in bone marrow but negative results in blood ($n = 5$) had a significantly shorter survival ($P = 0.0414$) than patients tested negative in both compartments ($n = 12$). One patient with a CK 20-negative result died postoperatively and therefore was excluded from calculation.

Discussion

It has been suggested (12, 13) that CK 20 mRNA detection by RT-PCR in bone marrow is a promising marker for disseminated tumor cells of solid carcinoma origin. In a previous study, we demonstrated that this holds true for colorectal carcinoma by analyzing the bone marrow of these patients (8). Here, we have extended our investigations to gastric and pancreatic carcinoma patients. In addition to bone marrow, we tested venous blood samples from these patients with a nested duplex CK 20 RT-PCR. Using the newly developed nested CK 20 RT-PCR refined on duplex approach, we went toward routine diagnosis. Although in the present study all patients with tumor recurrence and metachronous metastasis were excluded, the percentage of CK 20-positive samples decreased. The rate of CK 20-positive results in bone marrow of colorectal cancer patients was 20 of 65 (31%) compared to previously reported 20 of 57 (35%). A clear relationship between the detection rate and the progress of disease (stages I–IV) could be demonstrated. With respect to the CK 20 protein expression in gastric cancer tissue (6) and our findings of the CK 20 mRNA expression in 2 of 3 gastric cancer cell lines, we

Table 3 *Comparative analysis of corresponding bone marrow (bm) and venous blood (vb) from colorectal, gastric and pancreatic cancer patients with CK 20-specific nested RT-PCR according to the tumor stage*

Tumor samples	UICC classification (TNM[a] stage)	Paired samples	Equivalent results[b]		Divergent results[b]	
			bm+/vb+	bm−/vb−	bm+/vb−	bm−/vb+
Colorectal	Stage I	6		5	1	
$n = 39$	Stage II	13	1	11	1	
	Stage III	4		4		
	Stage IV	16	6	8	2	
	Σ	39	7	28	4	
Gastric	Stage I	4		4		
$n = 18$	Stage II	2		1	1	
	Stage III	4		4		
	Stage IV	8	1	4	3	
	Σ	18	1	13	4	
Pancreatic	Stage I–IV	13		12	1	
$n = 13$						

[a] TNM, tumor-node-metastasis.
[b] bm+/vb+, positive result in both aspirates; bm−/vb−, negative result in both aspirates; bm+/vb−, positive result in bm and negative result in corresponding vb; bm−/vb+, negative result in bm and positive result in vb.

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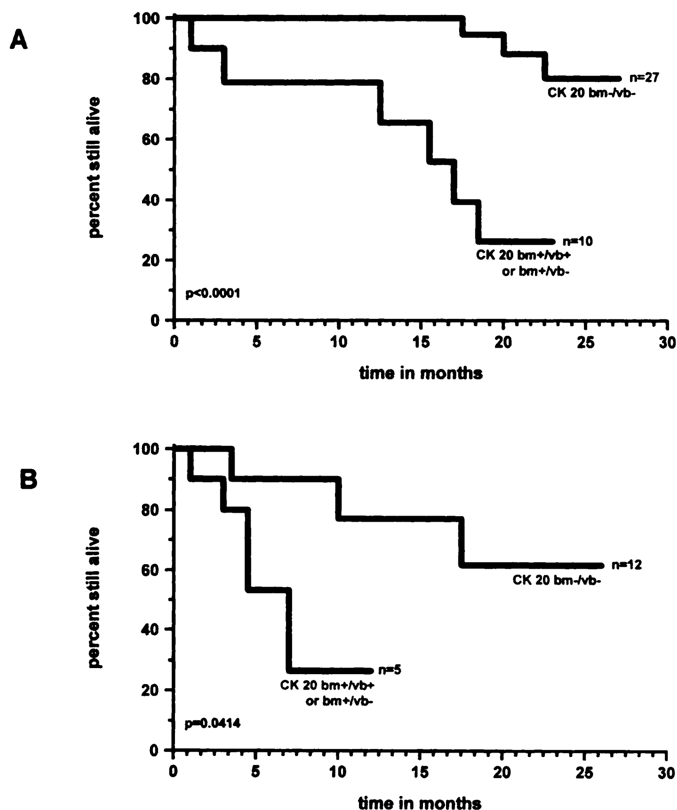

Fig. 3. Survival of patients tested in bone marrow (*bm*) and venous blood (*vb*) for CK 20 mRNA expression in parallel. Calculation was done with the Kaplan-Meier method. *A*, colorectal cancer patients with CK 20-positive results in both compartments or with positive results in bone marrow and negative results in venous blood ($n = 10$) were compared to CK 20-negative sets of samples ($n = 27$). *B*, gastric cancer patients with CK 20-positive results in both compartments or with positive results in bone marrow but negative results in venous blood ($n = 5$) were compared to CK 20-negative sets of samples ($n = 12$).

screened bone marrow isolates from patients suffering from this disease. The detection rate for CK 20 mRNA in these samples (22%) was lower than in the colorectal cancer group (31%). Only few reports deal with investigations of tumor cell dissemination in venous blood using molecular biological methods: circulating hepatocellular carcinoma cells from hepatocellular carcinoma patients (14) or prostate-specific antigen-synthesizing cells of patients with prostate cancer (15) are described. We addressed the question of whether this CK 20 RT-PCR assay is also applicable for venous blood specimens. Fifty-two blood samples obtained from colorectal cancer patients and 30 blood isolates from gastric cancer patients were screened. In both digestive tract diseases, the detection rate of disseminated epithelial cells was 17% in this body compartment. Again a stage-dependent detection rate was shown for colorectal cancer patients, while only suggested for the group of the gastric cancer patients. In contrast to our previous promising investigations of pancreatic carcinoma cell lines (7 of 8 positive), the detection rate in bone marrow ($n = 27$) and venous blood ($n = 22$) isolates of pancreatic cancer patient was not satisfactory. Investigation of a control panel of patients without apparent carcinoma yielded 2 of 22 positive bone marrow aspirates. These two patients were the previously reported familial adenomatous polyposis patient (8) and a female patient with liver adenoma type I with a tumor mass was about 1.5 kg. This liver adenoma patient also tested positive in the blood. Follow-up testing will show whether this positive detection will become negative after tumor resection. Another positive result in a blood isolate was obtained from a patient suffering from chronic pancreatitis who also participates in a clinical follow-up program. Overall, 2 of 58 control blood samples tested positive.

Comparative analyses of paired bone marrow and venous blood aspirates from the same patient are very rare. Investigations of neuroblastoma patients ($n = 14$) and more recently a study for CK 19 mRNA in breast cancer patients ($n = 13$) were reported (16, 17). We have investigated 70 sets of bone marrow and blood isolates from gastrointestinal cancer patients obtained prior to operation. Results obtained from the analysis of the sets of samples strongly suggest that in bone marrow and blood the same type of cells is detected. The detection rate in bone marrow compared to corresponding blood investigation was higher. It can be concluded that the survival conditions for tumor cells are superior to the conditions in peripheral blood and/or that the bone marrow may function as a filter. A very recent study on a larger group of prostate cancer patients investigated for disseminated disease by a prostate-specific antigen-specific RT-PCR in both body compartments came to a similar conclusion (18).

In summary, the results emphasize the importance of testing the bone marrow compartment. The clinical value of our findings with the CK 20 RT-PCR is shown by the clinical follow-up investigations of the patients, who were analyzed in both specimens. Colorectal and gastric cancer patients who tested positive for CK 20 mRNA in both bone marrow and venous blood or positive in bone marrow alone (but negative in blood) had a significantly shorter survival than the CK 20-negative patients. This is the first work to test an expanded panel of corresponding bone marrow and venous blood samples for disseminated gastrointestinal carcinoma cells by RT-PCR with clinical relevance.

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