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ORIGINAL ARTICLE

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Molecular detection of clinical colorectal cancer metastasis: how should multiple markers be put to use?

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Abstract *Background and aims:* Up to 45% of colorectal cancer (CRC) patients will develop local recurrence or metastasis following curative resection. The latter is due to cells shed from the primary carcinoma prior to or during surgery. The aim of this study was to contribute toward a "rational"-approach for detecting these disseminated tumor cells (DTC) using a combination of independent markers and detection methods. *Patients/methods:* Liver, lymph node, and bone marrow samples from 246 CRC patients were screened for DTC using three markers: mutated K-ras was detected by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP), and cytokeratin 20 (CK20) and guanylylcyclase C (GCC), indicating circulating epithelial cells, were tracked by nested reverse-transcription (RT) PCR. *Results:* The rate of positive findings of the individual markers (CK20: 88%; GCC: 88%; K-ras: 67%) and their combinations (88–50%) was significantly higher in biopsies from liver metastases than in liver samples from patients without evident distant metastasis (M0; $p<0.03$). The detection rate of individual markers (except GCC) was also significantly elevated in inconspicuous liver tissue adjacent to metastasis compared with specimens from M0 patients. When using the concomitant detection of all three markers as criterion for DTC in the liver of M0 patients, however, no patient was DTC-positive. Therefore, the concomitant presence of the two CEC markers (CK20 plus GCC) and/or the presence of mutated K-ras were preferred for a combined evaluation, which resulted in a 24% detection rate for biopsies from both liver lobes. This translates into 39% of M0 patients with at least one positive liver biopsy. *Conclusion:* Our results suggest that the concomitant detection of CK20 plus GCC and/or the presence of mutated K-ras are a rational approach for tracking CEC/DTC in CRC patients.

Keywords Cytokeratin 20 · Guanylylcyclase C · K-ras · Disseminated tumor cells · Circulating epithelial cells

Introduction

In colorectal cancer (CRC), histopathologic staging is still the most important predictor of prognosis [1]. However, up to 45% of patients without residual tumor load following resection (termed M0 by histopathology) develop local recurrence or metastasis [2, 3]. The latter is caused by cells spread from the carcinoma prior to or during surgery, which escape current staging methods [1]. In this context, detection of disseminated tumor cells (DTC), or circulating epithelial cells (CEC), may add information to the established staging system and could be helpful in

defining more precisely the individual risk. Therefore, numerous studies have attempted to find DTC/CEC in lymph nodes [4–15], peripheral [10, 16–23], central [24] or mesenteric [10, 18, 21, 23] venous blood, bone marrow [5, 6, 20, 24], peritoneal fluid [18, 23, 25], and liver specimens [5, 6, 26–28] from CRC patients. Despite intensive efforts, there is still no specific marker for cells shed from the primary CRC. Therefore, a wide variety of markers and methods has been used for DTC detection. These comprise immunohistochemical approaches with anti-(pan)-cytokeratin antibodies [8, 11, 12, 23, 28], detection of genetic alterations in tumor suppressor [10, 29] or oncogenes [6, 10, 14, 26, 29], as well as qualitative and quantitative reverse transcriptase polymerase chain reaction (RT-PCR) assays, which have been used preferentially. These RNA-based approaches concentrated predominantly on cytokeratin 20 (CK20) [5, 7–10, 12–14, 16, 17, 19–22, 24, 25], guanylylcyclase C (GCC) [4, 5, 13, 15, 16, 20, 30], and carcinoembryonic antigen (CEA) [7, 9, 11, 13, 15, 17–20, 25]. Since these mRNA species indicate CEC rather than DTC, specificity is still a matter of discussion as well as sensitivity and the absence of assay standardization [31, 32]. Of note, several studies detected CK20 [5, 15, 16, 19], GCC [15, 16, 20], and CEA mRNA [15, 19, 20, 33] in specimens from individuals without malignant disease. To overcome this lack of specificity in CRC patients, some studies simultaneously investigated two or more markers, or different detection techniques. These include the simultaneous investigation of two or more independent RT-PCR markers [5, 7, 9, 13, 15, 17–22, 25], combinations of RT-PCR and immunohistochemistry (IHC) assays [8, 11, 12], a combined analysis of K-ras and CK20 [14], and a joint detection using cytology and IHC [23]. As could be expected from this substantial variation, the definition of a "positive" test result differed remarkably. Some studies classified patients or samples as "positive" if any marker in any investigated tissue proved positive [18, 21–23], whereas others accepted samples as "positive" only if the result of a given marker was confirmed by another independent marker [17, 25].

The aim of this study was to make a contribution toward a rational approach of using a combination of independent markers and detection methods simultaneously. To that end, the mRNA-based markers CK20 and GCC and the DNA-based marker K-ras (defined by matching K-ras mutations in tumor and investigated tissue) were analyzed individually and in combination in samples from clinical metastasis and in inconspicuous matching tissue adjacent to these metastases. This comparison was to determine the quality of the marker panel in tissue that is visibly or microscopically affected by invading cancer cells. In addition, these results were compared with inconspicuous samples from patients without distant metastasis as well as with inconspicuous samples from all study patients. Finally, the incidence of CK20 and GCC mRNA was evaluated in the subgroup of liver and lymph node samples with K-ras mutated DTC.

Materials and methods

Patients and tissues

Two hundred and forty-six patients undergoing elective surgery for CRC between January 1998 and July 2001 were included in the study after they had given their informed consent. Surgery in this monocentric, prospective study was performed at the Municipal Hospital at Nuremberg (Department of Visceral, Thoracic, and Endocrine Surgery). Patient samples included bone marrow (aspirates from the iliacal crest), liver (biopsied from the left lobe [segment 3] and the right lobe [segment 5], mucosa (taken from the oral resection margin), and lymph nodes (taken from the para-aortic area), all clinically free of metastasis, as well as tumor (taken from the non-necrotic part). From 15 patients staged International Union Against Cancer (UICC) III or IV, 18 tissue samples were available with clinical metastases (16 liver biopsies, 2 lymph nodes) as well as 35 specimens from clinically inconspicuous matching adjacent tissue (28 liver biopsies, 7 lymph nodes). All tissues were immediately placed into cryovials, shock frozen in liquid nitrogen, and stored at −80°C until further processing.

RNA/DNA isolation

A combined RNA/DNA extraction was performed using the Qiagen RNA/DNA Kit (Qiagen, Hilden, Germany) according to the manufacturer's recommendations. In brief, solid tissue samples weighing about 100 mg were homogenized in lysis buffer. Bone marrow samples were thawed in lysis buffer, mixed, and then processed as the other specimens. The amount of isolated nucleic acids was determined spectrophotometrically.

Detection of K-ras mutations

A polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) assay was used for the detection of K-ras mutations in codons 12 and 13 as described previously [6, 27]. In short, DNA was amplified in the first PCR (30 cycles) with primers Ras A and Ras B (Table 1) generating an amplicon 166 bp long. By using the Ras A sense primer, a restriction site was introduced for the enzymes BstXI and XcmI. In the subsequent first digest, BstXI and XcmI cut the amplicon only if the first two bases of codons 12 or 13 were a wild type. An aliquot of the first digest served as a template

Table 1 Primer sequences. *CK20* cytokeratin 20, *GCC* guanylylcyclase C

Primer	Sequence
Ras A	5′-ACTGAATATAAACTTGTGGTCCATGGAGCT-3′
Ras B	5′-TTATCTGTATCAAAGAATGGTCCTGCACCA-3′
Ras C	5′-GGATGGTCCTCCACCAGTAATATGGATATTA-3′
Ras Seq	5′-ACCTCTATTGTTGGATCATA-3′
CK20 5′ outer	5′-TGGATTTCAGTCGCAGA-3′
CK20 3′ outer	5′-ATGTAGGGTTAGGTCATCAAAG-3′
CK20 5′ inner	5′-TCCAATCCAGACACACGGTGAACTATG-3′
CK20 3′ inner	5′-CAGGACACACCGAGCATTTTGCAG-3′
GCC 5′ outer	5′-TGGATTTCAGTCGCAGA-3′
GCC 3′ outer	5′-ATGTAGGGTTAGGTCATCAAAG-3′
GCC 5′ inner	5′-AATCAGCGTCCTGATGATGGGCAAC-3′
GCC 3′ inner	5′-ATGAGGACACAGCCCATCCGTTGTG-3′

for the second PCR (30 cycles) with primers Ras A and Ras C (Table 1). Primer Ras C introduced a restriction site for enzymes BstXI and XcmI in mutant as well as in wild-type amplicons. The resulting amplicon had a length of 152 bp and underwent a second restriction. The second digest was run on a polyacrylamide gel, stained with ethidium bromide and analyzed by UV-video-densitometry. Amplicons of mutated DNA were cut only once into fragments 134 bp and 18 bp long, whereas amplicons of wild-type DNA were cut twice into fragments 106, 28, and 18 bp long.

DNA sequencing

DNA sequencing was performed as described previously [6]. In brief, DNA bands 134 bp long were excised from agarose gels, purified using the Qia Quick gel extraction kit (Qiagen, Hilden, Germany), and subsequently amplified with the Big Dye Terminator Cycle Sequencing Ready Reaction Kit (ABI Prism, Weiterstadt, Germany) using primer Ras Seq (Table 1). The product of 110 bp was run on an ABI 310 Sequencer (ABI Prism, Weiterstadt, Germany).

Detection of CK20 and GCC mRNA

Detection of CK20 and GCC mRNA by RT-PCR was performed as described previously [5]. In short, reverse transcription of RNA into cDNA was performed using Sensiscript reverse transcriptase (Qiagen, Hilden, Germany) and the outer-3′-CK20 or GCC primers (Table 1). An aliquot of the RT mixture served as a template for the first PCR, which contained the primers for CK20 and GCC designated "outer" (Table 1). The first PCR generated fragments 558 (CK20) and 341 bp long (GCC). An aliquot of this reaction served as a template for the second, "nested" PCR that was performed with "inner" primers (Table 1). The second PCR generated fragments 291 bp (CK20) and 252 bp (GCC) long. The final PCR product was run on a 1.5% agarose gel, stained with ethidium bromide and analyzed by UV video densitometry. Samples displaying a fragment of expected length were assessed "positive," samples without such bands as "negative."

Detection limit and specificity of the RT-PCR and PCR-RFLP assays

The detection limit of all assays used in this study was determined by spiking experiments as described previously [5, 6]. For the PCR-RFLP assay (K-ras), a detection limit of one K-ras mutated cell in 10^4 background cells was established for routine use. However, in some samples an improved detection limit of one mutated cell in 10^6 wild-type cells was observed. The detection limit of the RT-PCR assays for CK20 and GCC was up to one CRC cell among 10^6 hematopoietic cells. As described previously [5, 27], samples from individuals without malignant disease were used to determine the specificity of all assays used in this study.

Statistics

The Chi-square test was applied to examine for the independent occurrence of investigated parameters. A p level ≤ 0.05 was considered significant.

Results

Patient characteristics

The characteristics of 246 CRC patients classified according to the K-ras status of their primary tumor are shown in Table 2. The mean age of all patients (140 males, 106 females) was 66.8 years. Patients with K-ras mutation in codons 12 and 13 of their primary CRC (n=92) had a mean age of 66.1 years and this age differed only slightly from that of patients with the K-ras wild-type sequence (67.2 years, n=153). From 15 patients (11 males, 4 females) staged UICC III or IV with a mean age of 64.4 years, tissue samples with clinical metastasis, as well as specimens from clinically inconspicuous matching adjacent tissue, were available. Sixteen patients (8 males, 8 females; mean age 65.1 years) with K-ras mutated primary CRC also harbored a matching K-ras mutation in clinically inconspicuous liver or lymph node samples.

According to the tumor-nodes-metastasis (TNM) classification, the majority of all tumors was staged T3 (63.7%). Distinctly lower rates were observed for tumor stages T2 (21.2%), T4 (12.2%), and T1 (2.9%). Tumor cell infiltration into regional lymph nodes (N1/2) was diagnosed by histopathology in 41%. Most patients were clinically free of distant metastasis (M0, 81.0%). After surgical resection, the vast majority of patients (82.1%) showed histopathologically no signs of residual tumor load (R0).

Incidence of CK20 and GCC in samples with clinical metastasis as well as in clinically inconspicuous specimens

Table 3 shows the incidence of CK20 and GCC mRNA in samples with clinical metastasis as well as in clinically inconspicuous specimens of various patient subgroups. For liver biopsies, the statistical analysis revealed significantly higher incidence rates in biopsies from clinical metastasis (CK20, 88%; GCC, 88%; CK20 + GCC, 81%) than in biopsies from CRC patients without distant metastasis (CK20 31%, p<0.00001; GCC 59%, p<0.03; CK20 + GCC 25%, p<0.0001), as well as in the group of all study patients (CK20 34%, p=0.00002; GCC 63%, p<0.05; CK20 + GCC 29%, p=0.00001). A significantly elevated ratio was also observed when comparing the incidences of CK20 or CK20 plus GCC in inconspicuous matching adjacent liver tissue (CK20 68%, CK20 + GCC 54%) with inconspicuous biopsies from patients of the M0 subgroup (CK20 31%, p<0.0001; CK20 + GCC 25%, p<0.001), as well as with all inconspicuous biopsies from all study patients (CK20 34%, p=0.0004; CK20 + GCC 29%, p=0.005). The small number of available metastatic lymph nodes (n=2) and adjacent inconspicuous samples (n=7) was too low for a meaningful evaluation. Never-

Table 2 Patient characteristics. *TNM* tumor-nodes-metastasis classification

	Total *n* (%)	Tumor ras+ *n* (%)	Tumor ras– *n* (%)
Number of patients (total)	246	92 (37.6)[c]	153 (62.4) [c]
Male	140 (56.9)	46 (33.1)[d]	93 (66.9) [d]
Female	106 (43.1)	46 (43.4)	60 (56.6)
Mean age in years (± standard deviation)	66.8 (10.8)	66.1 (9.9)	67.2 (11.3)
Number of patients with available samples of clinical metastasis an inconspicuous adjacent tissue	15	5 (33.3)	10 (66.7)
Male	11 (73.3)	5 (45.5)	6 (54.5)
Female	4 (26.7)	0 (0)	4 (100)
Mean age in years (± standard deviation)	64.4 (13.4)	59.7 (15.6)	67.6 (11.6)
Patients with K-ras mutated tissue samples	16	16 (100)	–
Male	8 (50)	8 (100)	–
Female	8 (50)	8 (100)	–
Mean age in years (± standard deviation)	65.1 (10.4)	65.1 (10.4)	–
TNM classification[a]			
T1	7 (2.9)	2 (2.2)	5 (3.3)
T2	52 (21.2)	18 (19.6)	34 (22.2)
T3	156 (63.7)	63 (68.5)	93 (60.8)
T4	30 (12.2)	9 (9.8)	21 (13.7)
N0	144 (59.0)	52 (57.2)	92 (60.1)
N1	51 (20.9)	21 (11.0)	30 (19.6)
N2	49 (20.1)	18 (19.8)	31 (20.3)
M0[b]	200 (81.0)	72 (78.3)	128 (83.7)
M1[b]	47 (19.0)	20 (21.7)	25 (16.3)
R0	201 (82.1)	72 (78.3)	129 (84.4)
R1	3 (1.2)	0 (0)	3 (2.0)
R2	41 (16.7)	20 (21.7)	21 (8.6)

[a] Based on 245 patients
[b] Based on 246 patients with 247 clinical diagnoses (one study patient with re-operation due to liver metastasis was included twice in the M classification with the old and new diagnosis)
[c] Based on 245 patients; no primary tumor available for 1 patient
[d] Based on 139 male patients; no primary tumor available for 1 patient

theless, both lymph node samples with clinical metastasis were positive for CK20 and GCC mRNA, whereas clinically inconspicuous adjacent lymph nodes showed lower CEC incidences (CK20 29%, GCC 14%, CK20 + GCC 14%) than inconspicuous samples from all study patients (CK20 79%, GCC 68%, CK20 + GCC 60%).

Incidence of K-ras versus the combination of K-ras, CK20, and GCC in samples with clinical metastasis and in clinically inconspicuous specimens

The incidence of mutated K-ras (defined as a DTC marker by a matching K-ras mutation in the primary CRC and the respective tissue sample), and the combined evaluation of this marker with CK20 and GCC, was compared in samples with clinical metastasis, as well as in clinically inconspicuous specimens (Table 4). The analysis in liver biopsies showed significantly higher incidences of mutated K-ras, as well as of the combination of all markers in biopsies from clinical metastasis (K-ras 67%, K-ras + CK20 + GCC 50%, K-ras and/or CK20 + GCC 88%) than in inconspicuous liver biopsies from M0 patients (K-ras 5%; K-ras + CK20 + GCC 0%; K-ras and/or CK20 + GCC 24%; each $p<0.00001$). A similar difference was observed when comparing the former with inconspicuous liver biopsies from all study patients (K-ras 8%, $p<0.00001$; K-ras + CK20 + GCC 6%, $p=0.00007$; K-ras and/or CK20 + GCC 27%, $p<0.00001$). When comparing the marker incidence in inconspicuous matching liver biopsies adjacent to metastasis (K-ras 30%, K-ras + CK20 + GCC 10%, K-ras and/or CK20 + GCC 57%) with that in inconspicuous biopsies of the M0 subgroup (K-ras 5%, K-ras + CK20 + GCC 0%, K-ras and/or CK20 + GCC 24%), a significant difference was observed ($p<0.001$ for all comparisons). The comparison of the respective results from all study patients (K-ras 8%, K-ras + CK20 + GCC 6%, K-ras and/or CK20 + GCC 27%) revealed a significant difference for K-ras ($p=0.017$), as well as for K-ras and/or CK20 plus GCC ($p=0.002$).

Coincidence of CEC markers in K-ras mutated liver biopsies and matching lymph node and bone marrow specimens of the same patient

The coincidence of the two CEC markers CK20 and GCC in K-ras mutated liver biopsies and in matching lymph node and bone marrow specimens of the same patient is

Table 3 Incidence of CK20 and GCC in samples with clinical metastasis as well as in clinically inconspicuous specimens

Marker	Clinical aspect	Liver biopsies[a, b]			Lymph node		
		N (total)	Positive *n* (%)	Negative *n* (%)	*N* (total)	Positive *n* (%)	Negative *n* (%)
CK20	Metastasis	16[c]	14 (88)	2 (12)	2	2 (100)	0 (0)
	Inconspicuous; adjacent tissue	28[d]	19 (68)	9 (32)	7[e]	2 (29)	5 (71)
	Inconspicuous; samples from M0 patients	333	104 (31)	229 (69)	202	157 (78)	45 (22)
	Inconspicuous; all tested samples	403	139 (34)	264 (66)	247	196 (79)	51 (21)
GCC	Metastasis	16[c]	14 (88)	2 (12)	2	2 (100)	0 (0)
	Inconspicuous; adjacent tissue	28[d]	17 (61)	11 (39)	7[d]	1 (14)	6 (86)
	Inconspicuous; samples from M0 patients	333	198 (59)	135 (41)	202	135 (67)	67 (33)
	Inconspicuous; all tested samples	403	255 (63)	148 (37)	247	168 (68)	79 (32)
CK20+GCC	Metastasis	16[c]	13 (81)	3 (19)	2	2 (100)	0 (0)
	Inconspicuous; adjacent tissue	28[d]	15 (54)	13 (46)	7[d]	1 (14)	6 (86)
	Inconspicuous; samples from M0 patients	333	82 (25)[e]	251 (75)	202	117 (58)	85 (42)
	Inconspicuous; all tested samples	403	115 (29)	288 (71)	247	149 (60)	98 (40)

[a] Results in biopsies with clinical metastasis compared with biopsies from M0 patients (CK20 $p<0.00001$, GCC $p<0.03$, CK20 + GCC $p<0.0001$) and compared with all inconspicuous biopsies tested (CK20 $p=0.00002$, GCC $p<0.05$, CK20 + GCC $p=0.00001$)
[b] Results in matching adjacent tissue compared with biopsies from M0 patients (CK20 $p<0.0001$, GCC $p=0.89$, CK20 + GCC $p<0.001$), as well as compared with all inconspicuous biopsies tested (CK20 $p=0.0004$, GCC $p=0.78$, CK20 + GCC $p=0.005$)
[c] Number of biopsies ($n=16$) based on patients ($n=13$) for whom biopsies with metastasis and matching inconspicuous adjacent tissue was available
[d] For 16 biopsies with clinical liver and 2 samples with lymph node metastasis, 28 adjacent, inconspicuous liver biopsies and 7 lymph node samples were available
[e] This number of biopsies (%) translates into 69/170 patients (41%) with at least one positive liver biopsy

Table 4 Incidence of K-ras versus the combination of K-ras plus CK20 plus GCC in samples with clinical metastasis as well as in clinically inconspicuous specimens

Marker	Clinical aspect	Liver biopsies[c, d]			Lymph node		
		N (total)	Positive *n* (%)	Negative *n* (%)	*N* (total)	Positive *n* (%)	Negative *n* (%)
K-ras[a]	Metastasis	6[e]	4 (67)	2 (33)	0	0 (0)	0 (0)
	Inconspicuous; adjacent tissue	10[f]	3 (30)	7 (70)	0	0 (0)	0 (0)
	Inconspicuous; samples from M0 patients	142	7 (5)	135 (95)	72	4 (6)	68 (94)
	Inconspicuous; all tested samples	180	14 (8)	166 (92)	92	7 (8)	85 (92)
K-ras + CK20 + GCC[b]	Metastasis	6[e]	3 (50)	3 (50)	0	0 (0)	0 (0)
	Inconspicuous; adjacent tissue	10[f]	1 (10)	9 (90)	0	0 (0)	0 (0)
	Inconspicuous; samples from M0 patients	111	0 (0)	111 (100)	72	2 (3)	70 (97)
	Inconspicuous; all tested samples	137	8 (6)	129 (94)	92	4 (4)	88 (96)
K-ras and/or CK20 + GCC	Metastasis	16	14 (88)	2 (12)	2	2 (100)	0 (0)
	Inconspicuous; adjacent tissue	28	16 (57)	12 (43)	7	1 (14)	6 (86)
	Inconspicuous; samples from M0 patients	364[g]	87 (24)[h]	277 (76)	202[g]	119 (59)	83 (41)
	Inconspicuous; all tested samples	446[g]	121 (27)	325 (73)	247[g]	152 (62)	95 (38)

[a] Based on patients with a K-ras mutation in the primary tumor ($n=92$)
[b] Based on patients with a K-ras mutation in the primary tumor and for whom RNA from liver biopsies ($n=77$ patients), lymph nodes ($n=92$ patients), and bone marrow ($n=44$ patients) was available
[c] Results of biopsies with clinical metastasis compared with all biopsies from M0 patients (K-ras, K-ras + CK20 + GCC, K-ras and/or CK20 + GCC each $p<0.00001$) and compared with all inconspicuous biopsies tested (K-ras $p<0.00001$, K-ras + CK20 + GCC $p=0.00007$, K-ras and/or CK20 + GCC $p<0.00001$)
[d] Results of matching adjacent tissue compared with biopsies from M0 patients (K-ras $p=0.00084$, K-ras + CK20 + GCC $p=0.00082$, K-ras and/or CK20 + GCC $p=0.0005$) and compared with all inconspicuous biopsies tested (K-ras $p=0.017$, K-ras + CK20 + GCC $p=0.59$, K-ras and/or CK20 + GCC $p=0.002$)
[e] Number of biopsies ($n=6$) based on patients ($n=5$) with a K-ras mutated primary tumor and for whom biopsies with metastasis and matching inconspicuous adjacent biopsies were available
[f] For 6 biopsies with clinical liver metastasis, 10 adjacent, inconspicuous liver biopsies were available
[g] Number of biopsies based on 189 patients (M0) and 221 patients (all)
[h] This number of biopsies translates into 74 out of 189 patients (39%) with at least one positive liver biopsy

Table 5 Coincidence of circulating epithelial cell (CEC) markers in K-ras-mutated liver biopsies and incidence of the respective markers in matching lymph node and bone marrow specimens of the same patient. *N* number of positive test results

	Total	K-ras *N* (%)	CK20 *N* (%)	GCC *N* (%)	CK20 + GCC *N* (%)	CK20 and/or GCC *N* (%)	K-ras and/or CK20 + GCC *N* (%)
Liver (evaluation based on patients)[a]	12	12 (100)	7 (63)[d]	8 (73)[d]	6 (55)[d]	9 (82)[d]	12 (100)
Liver (evaluation based on biopsies)[b]	23	15 (65)	6 (43)[e]	8 (57)[e]	5 (36)[e]	9 (64)[e]	18 (78)
Matching available lymph node samples	12	3 (25)	9 (75)	7 (58)	6 (50)	10 (83)	9 (75)
Matching available bone marrow samples[c]	5	0 (0)	1 (20)	0 (0)	0 (0)	1 (20)	1 (20)

[a] A patient was considered positive when at least one liver lobe was tested positive for the respective marker(s)
[b] Liver biopsy tested positive for the respective marker(s)
[c] No matching bone marrow samples available for 7 patients
[d] Based on the results from 11 patients; no RNA available for 1 patient
[e] Based on results of 14 K-ras-mutated liver biopsies, for which RNA was available

Table 6 Coincidence of CEC markers in K-ras-mutated lymph node samples and incidence of the respective markers in matching liver biopsies and bone marrow specimens of the same patient. *N* number of positive test results

	Total	K-ras *N* (%)	CK20 *N* (%)	GCC *N* (%)	CK20+GCC *N* (%)	CK20 and/or GCC *N* (%)	K-ras and/or CK20+ GCC *N*[a] (%)
Lymph node	7	7 (100)	6 (86)	5 (71)	4 (57)	7 (100)	7 (100)
Matching available liver biopsies (evaluation based on patients)[a]	7	3 (43)	5 (83)[d]	4 (67)[d]	4 (67)[d]	5 (83)[d]	6 (86)
Matching available liver biopsies (evaluation based on biopsies)[b]	14	4 (29)	8 (67)[e]	7 (58)[e]	6 (50)[e]	9 (75)[e]	8 (57)
Matching available bone marrow samples[c]	3	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)

[a] A patient was considered positive when at least one liver lobe was tested positive for the respective marker(s)
[b] Liver biopsy tested positive for the respective marker(s)
[c] No matching bone marrow samples available for 4 patients
[d] Based on the results from 6 patients; no RNA available for 1 patient
[e] Based on the results of 12 K-ras-mutated liver biopsies, for which RNA was available

shown in Table 5. The subgroup of patients with DTC-positive liver biopsies (defined by matching K-ras mutations in tumor and liver biopsy) was used to assess both the coincidence of the two CEC markers in the same liver biopsy, as well as the incidence of the whole marker panel in matching lymph nodes and bone marrow specimens of the same patient. From patients with DTC-positive liver, 63% were CK20 positive, 73% were positive for GCC, and 55% for both markers. In 82% of these patients, at least one marker was positive in the liver. On the basis of DTC-positive liver biopsies, 15 out of 23 biopsies (65%) of the 12-DTC positive patients harbored a K-ras mutation. Sixty-four percent of these mutated liver biopsies were found positive for at least one CEC marker. Separate and combined analysis revealed lower incidence rates for CK20 (43%) and GCC (57%), and for CK20 plus GCC (36%), but not for the combination of K-ras and/or CK20 plus GCC (78%). In 12 matching lymph node samples, separate and combined CEC incidences (CK20 75%, GCC 58%, CK20 plus GCC 50%, CK20 and/or GCC 83%), and the combination of K-ras and/or CK20 plus GCC (75%), prevailed clearly over the DTC incidence assessed by mutated K-ras (25%). For bone marrow specimens, the number of available samples and the DTC/CEC incidence was too low for meaningful evaluation.

Coincidence of CEC markers in K-ras-mutated lymph node samples and matching liver biopsies and bone marrow specimens of the same patient

The coincidence of the two CEC markers in K-ras-mutated lymph nodes, and in matching liver biopsies and bone marrow specimens of the same patient, is shown in Table 6. As for K-ras-mutated liver biopsies, the group of patients with DTC-positive lymph nodes was used to assess the coincidence of the CEC markers in the same lymph node sample, as well as the incidence of the whole marker panel in matching liver biopsies and bone marrow specimens of the same patient. Out of seven patients with DTC-positive lymph nodes, 6 (86%) were positive for CK20, 5 (71%) for GCC, and 4 (57%) for both markers. A

perfect match (100% coincidence) was obtained when the presence of at least one CEC marker was required. Analysis of matching biopsies on the basis of patients (n=7) showed a preponderance of individual and combined CEC marker incidences (CK20 83%, GCC 67%, CK20 plus GCC 67%, CK20 and/or GCC 83%), as well as of K-ras and/or CK20 plus GCC (86%) over the DTC incidence assessed by mutated K-ras (43%). The same was true of an evaluation on the basis of liver biopsies. Sixty-seven percent were positive for CK20, 58% for GCC, 50% for CK20 plus GCC, 75% for at least one marker, and 57% for K-ras and/or CK20 plus GCC, whereas only 29% of the biopsies harbored a K-ras mutation. For bone marrow specimens, again, the number of available samples and the DTC/CEC incidence was too low for meaningful evaluation.

Discussion

The aim of this study was to determine the diagnostic advantage and practicability of a multi-marker assay for the detection of metastatic cells spread from the primary CRC. For this purpose, the detection rate and coincidence of the CEC markers CK20 and GCC, and the DTC marker K-ras were assessed individually, and in combination in liver and lymph node samples with clinical metastasis, as well as in inconspicuous matching tissue adjacent to these metastases. These results were compared with inconspicuous liver and lymph node samples from patients without distant metastasis, as well as with respective samples from all study patients.

The sensitivity of the CEC markers (CK20 and GCC) had been determined in mucosa samples, which, due to their epithelial origin, express the two proteins. The sensitivity was 100% for both CK20 and GCC [5]. In addition, the detection rate was assessed in liver or lymph node metastases that were visible during surgery of the primary CRC. As expected, the rate of positive findings of the individual markers (88–67%) and their combinations (88–50%) was significantly higher in samples that were visibly affected by cancer cells than those in inconspicuous samples from patients without distant metastasis (M0), or all study patients. The observed lack of a perfect correlation between visible metastases and their detection by the marker panel used could be explained by the following reasoning:

1. Although the vast majority of tumors do express CK20 [34, 35] and GCC [20, 30], metastatic cells could have lost the expression of these markers. This is in line with results from Tot [36], who detected CK20 by IHC in only 17 out of 23 (74%) colorectal liver metastases
2. Primary CRCs harboring a K-ras mutation can be mosaic in this feature and a K-ras wild-type clone could have originated from the metastatic liver lesion [6, 37]
3. The number of viable tumor cells in samples could vary depending on factors such as tumor cell proliferation, rate of apoptotic cells, nutrient supply of metastatic cells, and extent of fibrotic tissue
4. Except for positive controls (mucosa and tumor samples), the assays used lack a 100% reliability [5]
5. Non-systematic, technical failures of the assays in question could have occurred. Additionally, when defining samples as “positive” only if two or more independent markers were present simultaneously, it can be deduced that the cumulative detection rate of the panel is lower or maximally equal to the respective lowest individual detection rate

When using the marker panel for detecting metastatic tumor cells, the incidence of the individual markers (CK20, K-ras, but not GCC) or their combinations was also significantly elevated in inconspicuous liver tissue adjacent to metastases compared with specimens from M0 patients. This indicates that the area containing DTC exceeds the clinically visible metastatic lesion. In that case, the detection of DTC/CEC markers in inconspicuous tissue adjacent to clinical metastasis could be helpful in defining a subgroup of patients who are at increased risk. Of course, this assumption has to be corroborated by a follow-up study. The clinical value of DTC detection in the liver is supported by a recent study, which correlated DTC tracking by mutated K-ras in inconspicuous liver biopsies, with reduced overall survival and increased risk of hepatic metastasis development [26]. In line with this, M1 patients showing a more generalized DTC infiltration of the liver had a significantly decreased median survival time compared with M1 patients affected by a localized or no hepatic DTC [38]. Additionally, Yokoyama and coworkers [28] tracked micro-metastases adjacent to clinical metastasis by IHC for CK20 and correlated positive immuno-staining with an increased risk of intra-hepatic recurrence after hepatic resection, and with poorer outcome.

Of course, the general aim of detecting CEC/DTC is a making a contribution toward a more individual staging of patients resected with curative intention (R0). It is interesting to note that the percentage of patients who suffer from metastatic recurrence, despite a preceding resection with curative intent, ranges from 33 to 50% [2, 39, 40]. Since the majority of these recurrences show systemic dissemination of the carcinoma (∼80% [39]), with the liver being the preferential site of metastatic spread, we are tempted to suppose that the simultaneous detection of the two CEC markers in M0 patients (41% [5]) reflects the upper margin of an estimated percentage of patients with liver recurrence. Such an estimate depends, of course, on the sensitivity and specificity of the markers used.

In a recent study, CK20 and GCC showed similarly high sensitivities of 93 and 97% in histologically involved lymph nodes, but differing specificities as indicated by detection rates of 47 and 13% in lymph nodes from pa-

tients without malignant disease [15]. These data are in line with our own findings, with detection rates of 61% (CK20) and 0% (GCC) in various tissue samples of patients with benign disease or healthy control participants [5]. Therefore, we assume that a diagnosis based on the presence of both markers, CK20 plus GCC, has increased specificity regarding CEC detection without losing too much of its sensitivity (88% vs. 81% for the combination). The high specificity is evidenced by the fact that none of 44 tissue samples available from participants without malignancy were CEC (CK20 plus GCC) positive [5]. The same criterion of positivity was proposed by another group who added CK20 to CEA in order to increase specificity [17, 25]. A further addition of mutated K-ras and a diagnosis based on the simultaneous presence of three markers, however, decreased the sensitivity of this combination to 50%. The low detection rate of this marker panel is also reflected by the absence of positive results in liver biopsies of M0 patients. This is highlighted when taking into consideration liver or lymph node samples with DTC infiltration proven by a K-ras mutation identical to that in the primary CRC. In lymph node samples the coincidence with CK20 (86%) was relatively near to that reported by another group (100% [14]), but was distinctly reduced to 57% if the simultaneous detection of both CEC markers was required. An even more pronounced decrease in agreement between mutated K-ras and the two CEC markers was found in liver biopsies. The reason for the relatively large disagreement between K-ras and the two CEC markers is not clear. We speculate that neoplastic cells that are both K-ras mutated and metastatic lose epithelial differentiation markers more easily than the respective cells without K-ras mutation. Others have speculated that the detection of mutated K-ras could be based on DNA fragments from necrotic or apoptotic cells [41], the RNA of which has been degraded. These speculations are in line with the findings of Clarke et al. [42], who found CK species in 7 out of 13 (54%) K-ras-mutated lymph node specimens.

The principle used in this study for interpreting the results from two or more markers has been described under the heading "believe the negative" [43]. This implies that a positive result of a given marker is nullified by a negative result of another marker determined concomitantly. The strength of that approach is that a positive result depends on the simultaneous detection of two or more markers, which can validate one another. A different approach has been coined "believe the positive." In this case, a patient or sample is considered positive if at least one marker of a series (in at least one of the samples investigated) turns out to be positive. So far the majority of published studies have followed the latter approach [9, 10, 18, 21–23]. For example, Guller et al. [18] tracked CEA and CK20 mRNA by quantitative RT-PCR in the peritoneal lavage fluid and peripheral and mesenteric blood of CRC patients staged M0. Interestingly, a positive result of at least one marker in any sample tested (28%) was of prognostic relevance. This is in partial contrast with findings published by Wharton et al. [19], who found significantly increased detection rates (74%) when applying the principle "believe the positive" in tests with two markers and multiple samples compared with using one marker and one sample (34% or 48% positive results).

To combat this problem, some groups investigated two or more markers, but performed the respective analysis on an individual basis for each independent marker instead of a combined evaluation [13, 15, 20]. The problem seems to be even more accentuated when combining different methods such as RT-PCR with IHC [8, 11]. To our knowledge, only Rosenberg et al. [12] found improved specificity and prognostic value for a combination of RT-PCR and IHC for CK20 compared with CK20 RT-PCR results alone.

The low sensitivity associated with using mutated K-ras plus the two CEC markers prompted us to combine two strategies: the combination of CK20 and GCC according to the principle "believe the negative" was maintained, since these markers apparently validate each other in a beneficial way. On this basis, mutated K-ras results from tissue samples were added according to the principle "believe the positive," since this marker is validated by an identical mutation in the primary CRC. The approach not only allows the inclusion of all patients, but also has sensitivity comparable to or even higher than that of the two CEC markers. For the M0 subgroup this translates into a 39% rate of patients with at least one CEC/DTC-positive liver biopsy. Of course, only a follow-up study will determine the value of this approach and whether or not these M0 patients constitute a subgroup at increased risk of metastatic liver recurrence.

In conclusion, the concomitant use of two CEC markers determined by the same method follows a rational approach, since they can validate one another. The addition of further markers determined by different methods can constitute a problem in terms of a too restrictive selection of patients if the principle "believe the negative" is followed. We, therefore, recommend adding a further marker according to the principle "believe the positive," provided that the marker in question can be corroborated as well. Based on the principle outlined above, an 88% detection rate was found for clinical liver metastases and 57% of inconspicuous liver biopsies adjacent to these metastases were positive for DTC/CEC. These rates were significantly higher than that obtained from biopsies of M0 patients (24%, $p<0.001$). The latter incidence translates into a 39% rate of CRC M0 patients with DTC/CEC affecting the liver, who could constitute a subgroup at increased risk of overt liver metastasis. A follow-up study will help to determine the clinical value of this approach.

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