

ORIGINAL ARTICLE

Limitations of cytokeratin 20 RT-PCR to detect disseminated tumour cells in blood and bone marrow of patients with colorectal cancer: expression in controls and downregulation in tumour tissue

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Aims: Despite informative staging of patients with colorectal cancer, some patients with localised disease at diagnosis will develop recurrence or metastasis. Attempts to improve staging include sensitive detection of disseminated tumour cells in blood and bone marrow by reverse transcriptase polymerase chain reaction (RT-PCR). The results of this study have been considered in relation to the controversial results in the literature to elucidate the usefulness of cytokeratin 20 (CK20) RT-PCR to detect disseminated tumour cells further.

Patients/Methods: Blood and bone marrow samples from 30 patients with colorectal cancer were studied by CK20 RT-PCR. Specificity was evaluated in 47 blood and 15 bone marrow samples from non-cancer controls. In addition, the expression of CK20 mRNA and protein was studied in normal and tumour colon tissue samples.

Results: CK20 expression was detected in nine of 30 and nine of 19 of the blood and bone marrow samples from patients with colorectal cancer, respectively. In non-cancer control blood and bone marrow samples, CK20 expression was detected in 10 of 47 and four of 15, respectively. A difference between patient and control samples may be observed in terms of frequency of positive PCR tests. In tissue samples, CK20 mRNA expression was downregulated in tumour compared with normal colon tissue.

Conclusions: CK20 expression was downregulated in tumour tissue compared with normal colon and a background expression of CK20 was seen in some control blood and bone marrow samples. Despite a lack of standardisation (which hampers comparison of studies), these results, together with other reports in the literature, suggest that CK20 may still be a suitable marker, but that background expression and threshold setting should be studied further.

The primary treatment of colorectal cancer is surgical resection. Accurate examination of the resected specimen and the precise detection of lymph node and distant metastasis is needed to determine the TNM stage, which is the best predictor of survival.^{1,2} Unfortunately, a large number of patients with early stage colorectal cancer, who have a localised tumour at diagnosis, will develop recurrent or metastatic disease,³ indicating that these patients had minimal residual disease after surgery. Several studies have analysed the presence and significance of disseminated tumour cells in blood, bone marrow, and lymph nodes.^{4–10} A limited number of studies show that the detection of disseminated tumour cells may lead to the improvement of staging and may contribute to a more accurate prediction of prognosis.^{11–16}

"In colorectal cancer, cytokeratin 20 is considered a useful marker, although its suitability is disputed"

Because of its high sensitivity, reverse transcriptase polymerase chain reaction (RT-PCR) based on the amplification of cell type specific mRNA is increasingly used to detect disseminated tumour cells. For these RT-PCR studies, an appropriate marker gene has to meet at least two requirements. First, the marker has to lack expression in tissues in which the disseminated tumour cells need to be detected, which refers to the specificity of the test. Second, adequate expression of the marker in tumour tissue is a prerequisite for

the sensitivity of the test.^{6–7,10,17} In colorectal cancer, cytokeratin 20 (CK20) is considered a useful marker,^{18,19} although its suitability is disputed.^{20–25} Some studies report a relation between CK20 mRNA detection in blood or bone marrow and stage of disease²⁶ or prognosis.^{14,27} However, other studies do not confirm these results, and both low specificity and low sensitivity are reported: transcripts were identified in blood and bone marrow samples of non-cancer controls^{20,23–25} or CK20 mRNA was almost solely identified in samples of patients with extensive disease²¹ or in no patient samples at all.²² Moreover, little is known about CK20 mRNA expression in colorectal tumours. Moll and colleagues²⁸ have studied CK20 protein expression and found that 95% of the colorectal tumours and metastases tested were CK20 positive. Interestingly, Wildi *et al* reported a strong CK20 protein signal in cancer cells within the tumour mass, but a downregulation of CK20 mRNA expression in the tumour compared with normal colon.²⁹

To elucidate the usefulness of CK20 RT-PCR to detect disseminated tumour cells further, we prospectively collected preoperative blood and bone marrow samples from a series of

Abbreviations: CK, cytokeratin; MNC, mononuclear cells; PBGD, porphobilinogen deaminase; PBS, phosphate buffered saline; RT-PCR, reverse transcriptase polymerase chain reaction

well characterised patients with colorectal cancer at different stages of disease. The specificity of the CK20 RT-PCR was studied in three groups of non-cancer controls: patients who underwent colorectal surgery for non-malignant diseases, patients without malignancy or colorectal disorder, and healthy donors. To study CK20 expression in colorectal tissue, we analysed the expression of CK20 protein and mRNA in normal colorectal mucosa and in colorectal tumour tissue.

MATERIALS AND METHODS

Patient and control material

In our study, we investigated 30 patients with histologically confirmed colorectal carcinoma, who were classified according to the UICC TNM classification³⁰: 15 patients were stage II, 10 stage III, and five stage IV. Peripheral venous blood samples (5–10 ml) were collected a few days before surgery from 30 patients, and bone marrow (3–8 ml) was aspirated from the anterior iliac crest of 19 patients at the time of surgery, just before the operation was started. Blood and bone marrow mononuclear cells (MNC) were isolated by density gradient centrifugation through Ficoll-Hypaque (Amersham Pharmacia Biotech, Uppsala, Sweden). MNC were washed twice with phosphate buffered saline (PBS), and cell pellets were snap frozen in liquid nitrogen and stored at -80°C until use. Fresh tumour and normal tissue samples were obtained from the resected specimen, snap frozen in liquid nitrogen, and stored at -80°C until use.

Blood samples of healthy donors ($n = 16$) and of patients suffering from non-malignant colorectal disorders ($n = 13$) (adenomatous polyps, Crohn's disease, and ulcerative colitis), and blood and bone marrow samples of patients without malignancy or colorectal disorder ($n = 18$) were used as negative controls. MNC were isolated from these samples as described above. The protocol was approved by the medical ethical committee and informed consent was obtained from each patient and healthy donor.

RNA extraction

Total RNA was extracted from the MNC pellets by TRIzol reagent (Life Technologies, Breda, The Netherlands) according to the manufacturer's instructions. This extraction method was also applied for RNA isolation from 10–20 frozen tissue sections of 20 μm thickness from 13 samples of normal colon mucosa and 18 samples of colorectal tumour. The measurement of RNA was performed by spectrophotometry at 260 nm.

Reverse transcription

An aliquot of 2 μg MNC RNA or 1 μg tissue RNA was preincubated with 250 pmol random hexamer primer (Roche Diagnostics GmbH, Penzberg, Germany) at 65°C for five minutes and immediately put on ice afterwards, where the other reagents were added. The RNA was then reverse transcribed in 20 μl RT buffer (50mM Tris/HCl, pH 8.3) containing 75mM KCl, 3.0mM MgCl₂, 10mM dithiothreitol, 200 μM of each nucleotide, and 200 U MMLV reverse transcriptase (Promega, Madison, Wisconsin, USA). This mixture was first incubated at 24°C for 10 minutes, then at 42°C for 60 minutes, and the reaction was terminated by heating at 95°C for five minutes.

Polymerase chain reaction of MNC samples

To monitor the quality of RNA and cDNA, we performed a PCR for the low copy housekeeping gene porphobilinogen deaminase (PBGD), using primers: 5'-CTGGTAACGGCAATGC GGCT-3' (sense) and 5'-GCAGATGGCTCCGATGGTGA-3' (antisense). The PCR for PBGD (339 bp) was performed with the following cycling protocol: denaturation at 94°C for three minutes, followed by 35 cycles of 45 seconds at 94°C , 60 seconds at 59°C , 90 seconds at 72°C , and a final extension at

72°C for 10 minutes. For the amplification of CK20 specific cDNA sequences we used primers identical to those published by Burchill and colleagues,¹⁸ namely: 5'-CAGACACACGGTG AACTATGG-3' (sense) and 5'-GATCAGCTTCCACTGTAGA CG-3' (antisense). The cycling protocol for CK20 (370 bp) consisted of: denaturation at 94°C for three minutes, followed by 40 cycles of 45 seconds at 94°C , 45 seconds at 60°C , 60 seconds at 72°C , and a final extension at 72°C for five minutes. All reactions were performed in a final volume of 50 μl PCR reaction mixture containing 75mM Tris/HCl, pH 9.0, 20mM (NH₄)₂SO₄, 0.01% Tween (wt/vol), 1.0mM MgCl₂ for PBGD or 1.5mM MgCl₂ for CK20, 200 μM of each nucleotide, 20 pmol of each PBGD primer or 15 pmol of each CK20 primer, 0.5 U thermostable DNA polymerase (Integro, Dieren, the Netherlands), and 2 μl cDNA. The reaction mixture was overlaid with mineral oil. For each cDNA sample, four individual CK20 PCR tests were performed. PCR products were analysed on a 2% agarose gel and visualised by ethidium bromide. To evaluate amplification specificity, reverse transcriptase negative control samples were included. No PCR products were detected in these controls. In addition, PCR products from 10 randomly chosen patient samples were sequenced and the correctness of the CK20 PCR product was confirmed.

Quality control aspects

Precautions were taken to avoid contamination. Procedures were carried out in separate rooms: RNA isolation and RT-PCR in a pre-PCR laboratory and the analysis of the PCR products in a post-PCR laboratory. Separate laboratory clothing was used in the different rooms. All materials were transported in a one way direction from the pre-PCR to the post-PCR laboratory only; a PCR product never reached the sample preparation laboratory. Contamination control samples were run in parallel with each experiment, in which RNA and cDNA were replaced by H₂O in the reverse transcription and the polymerase chain reaction, respectively.

In addition, we determined the sensitivity of the CK20 RT-PCR by analysing healthy donor blood to which cultured HT29 colon tumour cells were added in the following quantities: 0, one, two, five, 10, and 100 cells/ml blood. This experiment was performed twice, and four PCR tests were performed for each sample. In every experiment, external sensitivity control samples of one, two, and five HT29 cells/ml control blood were used to monitor the sensitivity of each individual experiment.

Polymerase chain reaction of tissue samples

First, PBGD PCR reactions were performed with various cDNA concentrations for each sample. Then, PBGD PCR was performed once again with a selected amount of cDNA, which resulted in an amount of PBGD product that was comparable with the PBGD products of the other samples. To analyse the comparability of PBGD expression more precisely, this second PBGD PCR was sampled after 24, 27, and 30 cycles. Subsequently, CK20 PCR was performed in duplicate using the selected amounts of cDNA. In this way, CK20 amplification was standardised for the amplification of PBGD in each sample. To determine the degree of expression of CK20 mRNA, the CK20 PCR reactions were sampled after 21, 24, 27, and 30 cycles. CK20 expression was semiquantified by scoring the amount of CK20 PCR product after 21, 24, 27, and 30 cycles.

Immunohistochemistry

Frozen tissue sections (4 μm) of 11 normal colon mucosa and 16 colorectal tumour samples were allowed to dry at room temperature, fixed in acetone for 10 minutes, and preincubated with normal horse serum for 20 minutes at room temperature. Then, sections of all tissue samples were incubated separately with anti-CK20 monoclonal antibodies K₅ 20.8

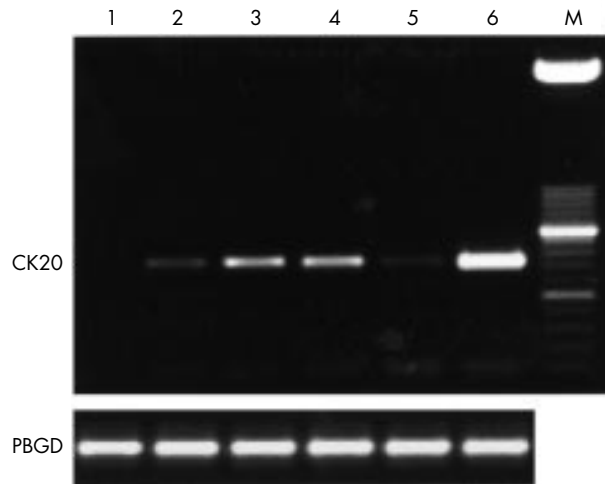


Figure 1 Sensitivity of the cytokeratin 20 (CK20) reverse transcriptase polymerase chain reaction (RT-PCR). CK20 and porphobilinogen deaminase (PBGD) PCR products of blood samples spiked with 0 (lane 1), one (lane 2), two (lane 3), five (lane 4), 10 (lane 5), and 100 (lane 6) HT29 cells/ml. M, 50 bp marker.

(Neomarkers, Fremont, California, USA) and K_{20.10} (Progen Biotechnik GmbH, Heidelberg, Germany) overnight at 4°C. Slides were washed with PBS and incubated with biotin conjugated antimouse antibody (Vectastain Elite ABC kit; Vector Laboratories, Burlingame, California, USA) for 30 minutes at room temperature. After the slides were washed, ABC complex (Vectastain Elite ABC kit) was applied for 45 minutes at room temperature. The slides were washed once more, the reactions were visualised with DAB, and the sections were counterstained with haematoxylin. All antibodies were diluted in PBS/1% bovine serum albumin and incubations were performed in a humidified chamber. CK20 expression was evaluated regarding two aspects: the percentage of positive epithelial or tumour cells and the intensity of staining.

RESULTS

Quality control analysis

In the sensitivity control experiments, in which we spiked healthy donor blood with HT29 cells, the lower limit of detection was one HT29 cell/ml (fig 1). Because the experiment was performed twice and four PCR tests were performed for each sample, in total eight PCR tests were analysed for each spiked concentration. Not all eight PCR tests of the blood samples spiked with one tumour cell/ml were CK20 positive; CK20 was detected in only three of the eight tests (sensitivity, 38%). In the blood samples spiked with two tumour cells/ml, CK20 expression was detected in seven of the eight PCR tests (sensitivity, 88%). All PCR tests performed on the blood samples spiked with five, 10, and 100 tumour cells/ml were CK20 positive (sensitivity, 100%). In the unspiked samples, no CK20 expression was detected (specificity, 100%). Along with each experiment, external sensitivity control samples of one, two, and five HT29 cells/ml blood were analysed, and all experiments reached the highest level of sensitivity. A PCR product was never detected in the H₂O control samples that were run along with each experiment. In all cDNA samples PBGD expression was detected, which confirms the high quality of the RNA and cDNA.

Blood and bone marrow samples

CK20 expression was studied in blood and bone marrow samples of non-cancer controls by means of four PCR reactions for each sample. CK20 mRNA was detected in 10 of the 47 blood samples and in four of the 15 bone marrow samples. Table 1 shows that none of the samples tested was consistently (four times) positive, and the detection of a PCR product is mostly restricted to one positive PCR for each sample.

We studied CK20 expression in 30 blood and 19 bone marrow samples from 30 patients with colorectal cancer. CK20 expression was detected in nine of the 30 blood samples (table 2) and in nine of the 19 bone marrow samples (table 3). None of the CK20 expressing samples was consistently (four times) positive; PCR products were detected in one (12 of 49), two (five of 49), or three (one of 49) of the four PCR tests. No relation was seen between CK20 detection and the stage of disease.

From 19 patients, both blood and bone marrow samples were analysed (table 4). In seven patients, both blood and

Table 1 Cytokeratin 20 (CK20) PCR of non-cancer control blood and bone marrow samples

	Number of positive tests out of four CK20 PCRs/sample					Positive/total patients
	0	1	2	3	4	
Blood	37	9	1	0	0	10/47
Healthy donor	13	3	0	0	0	3/16
Non-cancer colon patient	9	4	0	0	0	4/13
Non-cancer/non-colon patient	15	2	1	0	0	3/18
Bone marrow	11	4	0	0	0	4/15
Non-cancer colon patient	1	1	0	0	0	1/2
Non-cancer/non-colon patient	10	3	0	0	0	3/13

Table 2 Cytokeratin 20 (CK20) PCR of blood samples of patients with colorectal cancer

Stage	Number of positive tests out of four CK20 PCRs/sample					Positive/total patients
	0	1	2	3	4	
Stage II	10	3	2	0	0	5/15
Stage III	7	3	0	0	0	3/10
Stage IV	4	0	0	1	0	1/5

Table 3 Cytokeratin 20 (CK20) PCR of bone marrow samples of patients with colorectal cancer

	Number of positive tests out of four CK20 PCRs/sample					Positive/total patients
	0	1	2	3	4	
Stage II	3	4	2	0	0	6/9
Stage III	7	0	0	0	0	0/7
Stage IV	0	2	1	0	0	3/3

Table 4 Cytokeratin 20 (CK20) amplification in blood and bone marrow of the same patient with colorectal cancer

	Bone marrow	
	Positive	Negative
Blood		
Positive	2	3
Negative	7	7

bone marrow samples were negative, and in two patients both were positive. Blood and bone marrow results did not correspond in 10 patients: seven patients had CK20 positive bone marrow but negative blood samples, and three patients had CK20 positive blood but negative bone marrow samples.

Tissue samples

We studied the expression of CK20 protein and mRNA in 13 normal colon mucosal samples and 18 colorectal tumour samples (tables 5 and 6). Figure 2 shows CK20 mRNA expression in the various tissue samples and in HT29 tumour cells. Duplicate analysis showed identical results. By quantifying the expression of CK20 mRNA by attaching a value ranging from one to four to each sample on the basis of the amount of PCR product after 21, 24, 27, and 30 cycles (fig 2), we found that CK20 mRNA expression was low in colon tumour samples compared with normal colon mucosa samples. This difference is strengthened by the fact that the percentage of potentially CK20 expressing cells in tumour samples is higher than in normal samples (tables 5 and 6). In addition, fig 2 shows that

the degree of CK20 expression in most tumour tissues is comparable to that seen in HT29 cells, which were used to study the sensitivity of our assay, especially if we take into account the fact that tumour tissues are not made up entirely of tumour cells, whereas the cell line sample consists of 100% tumour cells. In contrast to the expression of mRNA, protein expression was similar in normal (table 5) and tumour (table 6) samples for both the percentage of positive cells and the intensity of staining. Some tumour samples showed partial loss of CK20 protein expression (fig 3A). The expression of CK20 protein was studied with two different monoclonal antibodies, K_s 20.8 and K_s 20.10. The staining pattern for both antibodies was identical. However, staining with K_s 20.10 was slightly more intense (fig 3B,C). There was no correlation between the degree of mRNA and protein expression as measured by the percentage of positive cells, intensity of staining, or a combination of these two (not shown).

DISCUSSION

RT-PCR is widely used to study the presence of small numbers of disseminated tumour cells in blood and bone marrow samples of patients with cancer.⁵⁻⁹ A possible marker for such a test in patients with colorectal cancer is CK20, the expression of which is almost entirely restricted to gastric and intestinal epithelium, urothelium, and Merkel cells.^{28 31 32} In our study, we performed an RT-PCR to detect CK20 positive cells in blood and bone marrow samples of patients with colorectal cancer. We tested the sensitivity of our assay in an in vitro system by adding HT29 colorectal tumour cells to healthy donor blood. The lower limit of detection was one tumour cell/ml blood. However, the rate of positive PCR tests varied among the various spiked concentrations. CK20 expression could be detected with a sensitivity of 100% (eight of eight) in the blood samples

Table 5 Cytokeratin 20 (CK20) mRNA and protein expression, measured by means of immunohistochemical staining with antibody K_s 20.10, in normal colon samples

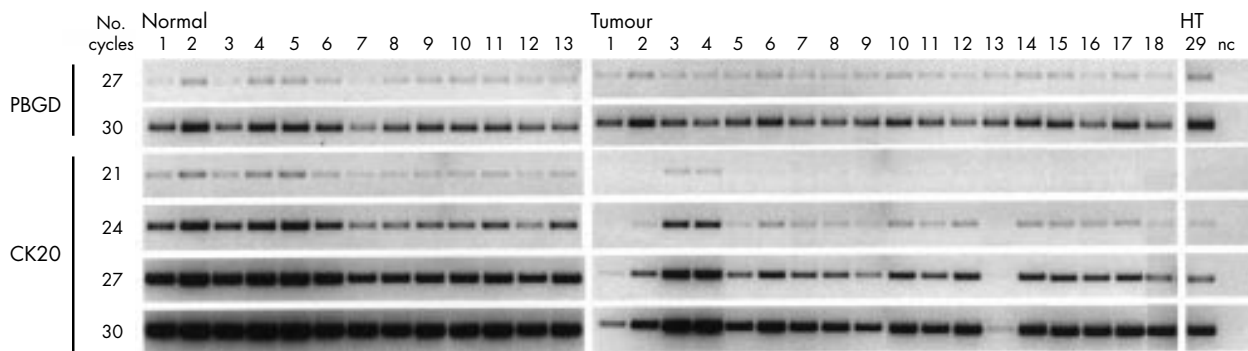
No.	Epithelial cells (%)	CK20 expression		
		mRNA	Protein	
			Positive cells	Intensity
1	40	4		
2	40	4	++	+++
3	25	4		
4	40	4	++	++
5	50	4	+++	+++
6	40	4	+++	+++
7	50	4	+++	+++
8	50	4	+++	+++
9	40	4	++	+++
10	60	4	+++	+++
11	60	4	+++	+++
12	40	4	+++	+++
13	40	4	+++	+++

No. refers to the corresponding number in fig 2. mRNA: 1, positive after 30 cycles; 2, positive after 27 cycles; 3, positive after 24 cycles; 4, positive after 21 cycles. Positive cells: +, <25% positive; ++, 25-75% positive; +++, >75% positive. Intensity: +, weak staining; ++, moderate staining; +++, strong staining.

Table 6 Cytokeratin 20 (CK20) mRNA and protein expression, measured by means of immunohistochemical staining with antibody K_s 20.10, in colorectal tumour samples

No.	Tumour cells (%)	CK20 expression		
		RNA	Protein	
			Positive cells	Intensity
1	40	2		
2	80	3	+++	+++
3	30	4	+++	+++
4	70	4		
5	60	3	+++	+++
6	70	3	+++	++
7	50	3	+++	+++
8	50	3	+++	+++
9	70	3	+	+++
10	70	3	++	+
11	80	3	+++	++
12	70	3	+++	+++
13	40	1	+	+++
14	60	3	++	+++
15	20	3	+++	++
16	60	3	++	++
17	50	3	+++	+++
18	80	3	++	+++

No. refers to the corresponding number in fig 2. mRNA: 1, positive after 30 cycles; 2, positive after 27 cycles; 3, positive after 24 cycles; 4, positive after 21 cycles. Positive cells: +, <25% positive; ++, 25–75% positive; +++, >75% positive. Intensity: +, weak staining; ++, moderate staining; +++, strong staining.

**Figure 2** Cytokeratin 20 (CK20) mRNA expression in normal colon and colorectal tumour samples. Porphobilinogen deaminase (PBGD) expression after 27 and 30 PCR cycles and CK20 expression after 21, 24, 27, and 30 PCR cycles in 13 normal colon and 18 colorectal tumour samples. The numbers of the normal (1–13) and tumour samples (1–18) correspond with the numbers used in tables 5 and 6, respectively. nc, H₂O negative control.

spiked with five, 10, and 100 tumour cells/ml, with a sensitivity of 88% (seven of eight) in the blood samples spiked with two tumour cells/ml, and with a sensitivity of 38% (three of eight) in the blood samples spiked with one tumour cell/ml. No CK20 expression was detected in the unspiked samples, which gives 100% specificity in these experiments. Thus, these results show that there is a correlation between the number of spiked tumour cells and the rate of CK20 positive PCR tests.

“Perhaps a threshold that regards a patient sample as positive if CK20 is amplified in at least two of four PCR reactions could be used to distinguish between background CK20 expression and CK20 expression resulting from tumour cells”

Using this assay, we detected CK20 mRNA in nine of the 30 blood samples (table 2) and in nine of the 19 bone marrow samples (table 3) from patients with colorectal cancer. In this small series of patients, neither CK20 expression in blood nor in bone marrow correlated with the stage of disease. Unfortun-

nately, CK20 mRNA was also detected in 10 of the 47 and four of the 15 control blood and bone marrow samples, respectively (table 1). However, the detection of CK20 expression in the control samples was mostly restricted to one of the four PCR tests; only in one of the 47 blood samples was CK20 mRNA detected in more than one PCR. This, in combination with the correlation between the number of spiked tumour cells and rate of CK20 positive tests, may lay the foundation for a threshold. Perhaps a threshold that regards a patient sample as positive if CK20 is amplified in at least two of four PCR reactions could be used to distinguish between background CK20 expression and CK20 expression resulting from tumour cells.³³ Despite the limited number of patients who underwent a potentially curative resection (n = 25) and the relatively short median follow up period of 20 months, we studied tumour recurrence in patients applying this threshold. The two patients with a CK20 positive preoperative blood sample developed distant metastases within 12 and 15 months of surgery. The two patients who had a positive preoperative bone marrow sample remained disease free after a follow up period of 19 months after surgery. Of the 20 patients who had

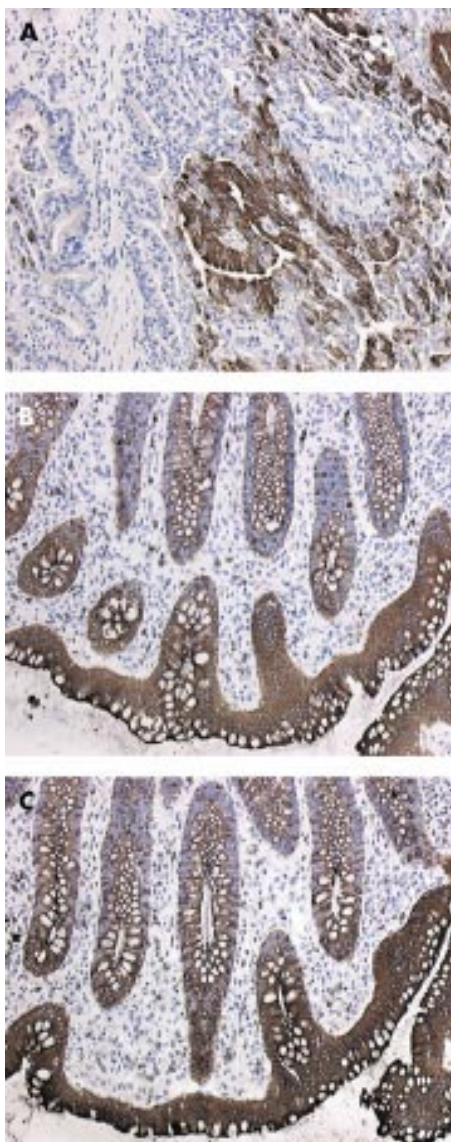


Figure 3 Cytokeratin 20 (CK20) protein expression in normal colon mucosa and colorectal tumour. (A) Colorectal tumour (number 9 in fig 2 and table 6) stained with the anti-CK20 monoclonal antibody K_{20.10}. Note that part of the tumour is negative. Normal colon mucosa (number 8 in fig 2 and table 5) stained with the anti-CK20 monoclonal antibodies (B) K_{20.8} and (C) K_{20.10}. Note that staining patterns are identical, but that staining with K_{20.10} is slightly more intense.

CK20 negative blood or bone marrow samples, only one patient developed distant metastases within 12 months after surgery, three patients developed local recurrence within six, 11, and 12 months, and 16 patients remained disease free for a median follow up period of 22 months, with a range of 13 to 32 months. This suggests that if this threshold is used, CK20 positive blood might predict distant metastasis. However, the examination of more patients and longer follow up of these patients is needed to give a decisive answer as to whether such a threshold might be suitable to predict the recurrence of disease.

The results in the literature regarding the detection of CK20 mRNA in blood and bone marrow of non-cancer controls are controversial. In accordance with our results, CK20 expression in control samples has been described in several other studies.^{20 23–25} There are several possible explanations for these unexpected positive results in control samples. The first explanation may be the illegitimate transcription of CK20 in

haematopoietic cells.^{17 23 34–37} Chelly *et al* suggested that a low amount of tissue specific gene transcription, in the order of one transcript/500–1000 cells, is present in non-specific tissues as the result of minimal activation of promoters by ubiquitous transcription factors in the absence of tissue specific transcription factors.³⁶ In this regard, Jung *et al* studied CK20 mRNA expression in two different white blood cell fractions separated by density gradient centrifugation: mononuclear cells and granulocytes.²⁵ They detected a background amount of CK20 mRNA expression in normal granulocytes only, and concluded that CK20 mRNA expression is not restricted to gastric and intestinal epithelium, urothelium, and Merkel cells. This may partly explain the CK20 mRNA expression in negative control samples, because some studies have used RNA isolated from the total white blood cell fraction to detect circulating tumour cells.^{20 24} However, it does not explain our results or the results of Champelovier and colleagues²³ or Litle and co-workers³⁸ because in these studies granulocytes were removed by density gradient centrifugation.

A second explanation may be the induction of a low amount of transcription of non-haematopoietic tissue specific genes in haematopoietic cells as a result of exposure to cytokines or growth factors. This has been described for the marker genes carcinoembryonic antigen and CK19 *in vitro* and *in vivo*.³⁹ However, CK20 mRNA expression in haematopoietic cells was not influenced by cytokines or growth factors when tested in the same *in vitro* test system.²⁵ Therefore, induction by cytokines is unlikely to explain CK20 expression in controls, although it cannot be ruled out.

A third explanation for the detection of CK20 mRNA in non-cancer control blood and bone marrow samples may be the presence of non-tumour epithelial cells that express CK20 mRNA. In this regard, it has been shown that circulating epithelial cells are detected in about 10% of the blood samples of patients undergoing bowel resection for benign conditions.⁴⁰ Our results suggest that CK20 is detected more frequently in blood and bone marrow samples of patients with non-malignant colorectal disorders compared with the other control samples (table 1); however, the number of patients studied is small. Unfortunately, CK20 is a differentiation marker and is therefore not suitable for discriminating between cancer cells and non-cancer cells.²⁸ No definite cancer related marker is available yet to detect colorectal carcinoma cells.

In contrast, there are several studies showing that CK20 expression is not seen in the blood and bone marrow of non-cancer controls.^{21–22 27 41–45} It is remarkable that in some of these studies the total white blood cell fraction was used to detect the disseminated tumour cells,^{21–22 27 42 44–45} because this fraction would be expected to contain CK20 gene transcripts as a result of the granulocytes present. A limited number of studies based on the analysis of larger series of patient samples demonstrated that the presence of disseminated tumour cells in blood and bone marrow detected by CK20 RT-PCR is related to the stage of disease or the prognosis of patients with colorectal cancer.^{14 26} The number of patient samples tested in our study is too small to evaluate the relation between the detection of CK20 mRNA expression and the stage of disease and clinical outcome of the patients.

A true comparison between the various studies to elucidate the divergent results regarding CK20 expression is difficult because of the lack of standardisation of the techniques across laboratories.^{23 33 46–49} Obvious differences in methods are the selection of the PCR primers,⁵⁰ the number of PCR cycles,³⁴ the number of PCR reactions performed for each sample, and the interpretation of conflicting PCR results.^{14 23 25 49 51} All these factors have an impact on the assay sensitivity and specificity and will therefore influence the results.

Despite our high assay sensitivity of one HT29 tumour cell/ml blood, which is comparable to other studies,^{14 26 45} our

results show a low consistency of positive CK20 PCR reactions within patient samples, even in samples of stage IV patients. When we consider the 18 patient samples in which we detected CK20 expression, more than half of the samples were positive for only one of the four PCR reactions, and no samples were positive for all four PCR reactions. Because the reproducibility of positive PCR results is dependent on the concentration of the marker used, the detection of disseminated tumour cells seems to be hampered by a low number of marker transcripts. Amplification of transcripts by RT-PCR is therefore a matter of chance, as has been reported by others,^{23 49 51-53} and is shown by our results. Thus, we believe that the numbers of CK20 transcripts in the blood and bone marrow of patients with colorectal cancer are often very low and cannot always be detected using our RT-PCR analysis.

“A true comparison between the various studies to elucidate the divergent results regarding CK20 expression is difficult because of the lack of standardisation of the techniques across laboratories”

Given the low number of CK20 transcripts in blood and bone marrow, another factor that has to be considered is CK20 mRNA expression in tumour tissue.¹⁷ Regarding CK20 protein expression, Moll and colleagues²⁸ reported expression in 95% of colorectal tumours and metastases. CK20 mRNA has been detected in most of the primary colon tumours and liver metastases; however, the number of tested samples was low and a highly sensitive CK20 RT-PCR was used.⁴⁵ Another study showed that the CK20 protein is expressed, but that the mRNA is downregulated in colorectal tumour tissue compared with normal colon mucosa.²⁹ Our data on CK20 mRNA expression in 13 normal and 18 tumour samples support these results by showing a lower degree of CK20 mRNA expression in tumour tissue compared with normal mucosa (fig 2). The simple semiquantitative method used in our study enabled us to compare the relative amounts of CK20 expression in normal colon mucosa and colorectal tumour samples. The use of more advanced quantification techniques, such as real time PCR, would have resulted in more detailed information. However, for our purpose, a semiquantitative determination of the CK20 mRNA expression was sufficient. In addition, the expression of the CK20 protein does not appear to be downregulated in tumour tissues compared with normal tissues (table 5 and 6). Thus, downregulation or heterogeneity of CK20 mRNA expression in the tumour cells (or both) could be one of the underlying reasons for the low numbers of marker molecules that we encountered in the RT-PCR analysis of blood and bone marrow samples of patients with (advanced stage) colorectal cancer.

We encountered some problems using CK20 RT-PCR to detect disseminated tumour cells in the blood and bone marrow of patients with colorectal cancer. We observed a downregulation of CK20 mRNA expression in colon tumour tissue compared with normal colon mucosa. However, in all tumour samples tested, CK20 mRNA expression could be detected. In our hands, the specificity of the CK20 RT-PCR was limited because CK20 mRNA expression was detected in blood and bone marrow samples of non-cancer controls. However, the degree of expression in some patient samples may be higher than the background expression seen in the control samples. Together with previously published results showing that CK20 can be used successfully as a marker to detect disseminated tumour cells, we conclude that CK20 may be a suitable marker, but its use is not without problems. More standardisation of the methods used and further research are needed to explain conflicting results in the literature and background expression in MNC. Furthermore, the possibility of setting a threshold, which could distinguish between background expression and increased expression as a result of dis-

Take home messages

- The expression of cytokeratin 20 (CK20) was lower in tumour tissue than in normal colon
- Background expression of CK20 was seen in some control blood and bone marrow samples, which limits the specificity of this method
- These results suggest that CK20 may still be a suitable marker to detect disseminated tumour cells in colorectal cancer
- However, background expression and threshold setting should be studied further

seminated tumour cells present in the blood and bone marrow of tumour bearing patients, needs to be examined.

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REFERENCES

- 1 **Bosman FT**. Prognostic value of pathological characteristics of colorectal cancer. *Eur J Cancer* 1995;**31A**:1216-21.
- 2 **Northover J**. The use of prognostic markers in surgery for colorectal cancer. *Eur J Cancer* 1995;**31A**:1207-9.
- 3 **Deans GT**, Parks TG, Rowlands BJ, *et al*. Prognostic factors in colorectal cancer. *Br J Surg* 1992;**79**:608-13.
- 4 **Pelkey TJ**, Frierson-HF J, Bruns DE. Molecular and immunological detection of circulating tumor cells and micrometastases from solid tumors. *Clin Chem* 1996;**42**:1369-81.
- 5 **Johnson PWM**, Burchill SA, Selby PJ. The molecular deletion of circulating tumour cells. *Br J Cancer* 1995;**72**:268-76.
- 6 **Bustin SA**, Dorudi S. Molecular assessment of tumour stage and disease recurrence using PCR-based assays. *Mol Med Today* 1998;**4**:389-96.
- 7 **Ghossein RA**, Bhattacharya S, Rosai J. Molecular detection of micrometastases and circulating tumor cells in solid tumors. *Clin Cancer Res* 1999;**5**:1950-60.
- 8 **Liefers GJ**, Tollenaar RA, Cleton JA. Molecular detection of minimal residual disease in colorectal and breast cancer. *Histopathology* 1999;**34**:385-90.
- 9 **Pantel K**, Cote RJ, Fodstad O. Detection and clinical importance of micrometastatic disease. *J Natl Cancer Inst* 1999;**91**:1113-24.
- 10 **Pantel K**, Ahr A. Immunocytochemical and molecular strategies for the detection of micrometastases in patients with solid epithelial tumours: a review. *Nucl Med Commun* 1998;**19**:521-7.
- 11 **Lindemann F**, Schlimok G, Dirschedl P, *et al*. Prognostic significance of micrometastatic tumour cells in bone marrow of colorectal cancer patients. *Lancet* 1992;**340**:685-9.
- 12 **Wood-DP J**, Banerjee M. Presence of circulating prostate cells in the bone marrow of patients undergoing radical prostatectomy is predictive of disease-free survival. *J Clin Oncol* 1997;**15**:3451-7.
- 13 **Kunter U**, Buer J, Probst M, *et al*. Peripheral blood tyrosinase messenger RNA detection and survival in malignant melanoma [see comments]. *J Natl Cancer Inst* 1996;**88**:590-4.
- 14 **Soeth E**, Vogel I, Roder C, *et al*. Comparative analysis of bone marrow and venous blood isolates from gastrointestinal cancer patients for the detection of disseminated tumor cells using reverse transcription PCR. *Cancer Res* 1997;**57**:3106-10.
- 15 **Liefers GJ**, Cleton JA, van de Velde CJ, *et al*. Micrometastases and survival in stage II colorectal cancer [see comments]. *N Engl J Med* 1998;**339**:223-8.
- 16 **Schlimok G**, Funke I, Bock B, *et al*. Epithelial tumor cells in bone marrow of patients with colorectal cancer: immunocytochemical detection, phenotypic characterization, and prognostic significance. *J Clin Oncol* 1990;**8**:831-7.
- 17 **Lambrechts AC**, van't Veer IJ, Rodenhuis S. The detection of minimal numbers of contaminating epithelial tumor cells in blood or bone marrow: use, limitations and future of RNA-based methods. *Ann Oncol* 1998;**9**:1269-76.
- 18 **Burchill SA**, Bradbury MF, Pittman K, *et al*. Detection of epithelial cancer in peripheral blood by reverse transcriptase-polymerase chain reaction. *Br J Cancer* 1995;**71**:278-81.

- 19 Soeth E, Röder C, Juhl H, *et al*. The detection of disseminated tumor cells in bone marrow from colorectal-cancer patients by a cytokeratin-20-specific nested reverse-transcriptase-polymerase-chain reaction is related to the stage of disease. *Int J Cancer* 1996;**69**:278–82.
- 20 Le Maire V, Sales JP, Wind P, *et al*. Evaluation et intérêt de nouveaux marqueurs moléculaires en cancérologie colique. *Ann Pharm Fr* 1998;**56**:9–17.
- 21 Chausovsky G, Luchansky M, Figer A, *et al*. Expression of cytokeratin 20 in the blood of patients with disseminated carcinoma of the pancreas, colon, stomach, and lung. *Cancer* 1999;**86**:2398–405.
- 22 Gunn J, McCall JL, Yun K, *et al*. Detection of micrometastases in colorectal cancer patients by K19 and K20 reverse-transcription polymerase chain reaction. *Lab Invest* 1996;**75**:611–16.
- 23 Champelovier P, Mongelard F, Seigneurin D. CK20 gene expression: technical limits for the detection of circulating tumor cells. *Anticancer Res* 1999;**19**:2073–8.
- 24 Bustin SA, Gyselman VG, Williams NS, *et al*. Detection of cytokeratins 19/20 and guanylyl cyclase C in peripheral blood of colorectal cancer patients. *Br J Cancer* 1999;**79**:1813–20.
- 25 Jung R, Petersen K, Kruger W, *et al*. Detection of micrometastasis by cytokeratin 20 RT-PCR is limited due to stable background transcription in granulocytes. *Br J Cancer* 1999;**81**:870–3.
- 26 Weitz J, Kienle P, Lacroix J, *et al*. Dissemination of tumor cells in patients undergoing surgery for colorectal cancer. *Clin Cancer Res* 1998;**4**:343–8.
- 27 Funaki NO, Tanaka J, Ohshio G, *et al*. Cytokeratin 20 mRNA in peripheral venous blood of colorectal carcinoma patients. *Br J Cancer* 1998;**77**:1327–32.
- 28 Moll R, Löwe A, Laufer J, *et al*. Cytokeratin 20 in human carcinomas. *Am J Pathol* 1992;**140**:427–47.
- 29 Wildi S, Kleeff J, Maruyama H, *et al*. Characterization of cytokeratin 20 expression in pancreatic and colorectal cancer. *Clin Cancer Res* 1999;**5**:2840–7.
- 30 Sobin LH, Wittekind Ch, eds. *UICC TNM classification of malignant tumours*, 5th ed. New York: John Wiley & Sons, 1997.
- 31 Miettinen M. Keratin 20: immunohistochemical marker for gastrointestinal, urothelial, and Merkel cell carcinomas. *Mod Pathol* 1995;**8**:384–8.
- 32 Moll R, Zimbelmann R, Goldschmidt MD, *et al*. The human gene encoding cytokeratin 20 and its expression during fetal development and in gastrointestinal carcinomas. *Differentiation* 1993;**53**:75–93.
- 33 van Houten VMM, Tabor MP, van den Brekel MWM, *et al*. Molecular assays for the diagnosis of minimal residual head-and-neck cancer: methods, reliability, pitfalls, and solutions. *Clin Cancer Res* 2000;**6**:3803–16.
- 34 Ko Y, Klinz M, Tatzke G, *et al*. Limitations of the reverse transcription-polymerase chain reaction method for the detection of carcinoembryonic antigen-positive tumor cells in peripheral blood. *Clin Cancer Res* 1998;**4**:2141–6.
- 35 Zippelius P, Kufer P, Honold G, *et al*. Limitations of reverse-transcriptase polymerase chain reaction analyses for detection of micrometastatic epithelial cancer cells in bone marrow. *J Clin Oncol* 1997;**15**:2701–8.
- 36 Chelly J, Concorde JP, Kaplan JC, *et al*. Illegitimate transcription: transcription of any gene in any cell type. *Proc Natl Acad Sci U S A* 1989;**86**:2617–21.
- 37 Sarkar G, Sommer SS. Access to a messenger RNA sequence or its protein product is not limited by tissue or species specificity [see comments]. *Science* 1989;**244**:331–4.
- 38 Little VR, Warren RS, Moore II D, *et al*. Molecular cytogenetic analysis of cytokeratin 20-labeled cells in primary tumors and bone marrow aspirates from colorectal carcinoma patients. *Cancer* 1997;**79**:1664–70.
- 39 Jung R, Kruger W, Hosch S, *et al*. Specificity of reverse transcriptase polymerase chain reaction assays designed for the detection of circulating cancer cells is influenced by cytokines in vivo and in vitro. *Br J Cancer* 1998;**78**:1194–8.
- 40 Hardingham JE, Hewett PJ, Sage RE, *et al*. Molecular detection of blood-borne epithelial cells in colorectal cancer patients and in patients with benign bowel disease. *Int J Cancer* 2000;**89**:8–13.
- 41 Funaki NO, Tanaka J, Sugiyama T, *et al*. Perioperative quantitative analysis of cytokeratin 20 mRNA in peripheral venous blood of patients with colorectal adenocarcinoma. *Oncol Rep* 2000;**7**:271–6.
- 42 Kawamata H, Uchida D, Nakashiro K, *et al*. Haematogenous cytokeratin 20 mRNA as a predictive marker for recurrence in oral cancer patients. *Br J Cancer* 1999;**80**:448–52.
- 43 Fujii Y, Kageyama Y, Kawakami S, *et al*. Detection of disseminated urothelial cancer cells in peripheral venous blood by a cytokeratin 20-specific nested reverse transcriptase-polymerase chain reaction. *Jpn J Cancer Res* 1999;**90**:753–7.
- 44 Nakamori S, Kameyama M, Furukawa H, *et al*. Genetic detection of colorectal cancer cells in circulation and lymph nodes. *Dis Colon Rectum* 1997;**40**:s29–36.
- 45 Wyld DK, Selby P, Perren TJ, *et al*. Detection of colorectal cancer cells in peripheral blood by reverse-transcriptase polymerase chain reaction for cytokeratin 20. *Int J Cancer* 1998;**79**:288–93.
- 46 Neumaier M, Braun A, Wagener C. Fundamentals of quality assessment of molecular amplification methods in clinical diagnostics. *Clin Chem* 1998;**44**:12–26.
- 47 Zippelius A, Lutterbüse R, Riethmüller G, *et al*. Analytical variables of reverse transcription-polymerase chain reaction-based detection of disseminated prostate cancer cells. *Clin Cancer Res* 2000;**6**:2741–50.
- 48 Keilholz U, Willhauck M, Rimoldi D, *et al*. Reliability of reverse transcription-polymerase chain reaction (RT-PCR)-based assays for the detection of circulating tumour cells: a quality-assurance initiative of the EORTC melanoma cooperative group. *Eur J Cancer* 1998;**34**:750–3.
- 49 Jung R, Ahmad-Nejad P, Wimmer M, *et al*. Quality management and influential factors for the detection of single metastatic cancer cells by reverse transcriptase polymerase chain reaction. *Eur J Clin Chem Clin Biochem* 1997;**35**:3–10.
- 50 He Q, Marjamaki M, Soini H, *et al*. Primers are decisive for sensitivity of PCR. *Biotechniques* 1994;**17**:82–7.
- 51 de Vries TJ, Fourkour A, Punt CJ, *et al*. Reproducibility of detection of tyrosinase and MART-1 transcripts in the peripheral blood of melanoma patients: a quality control study using real-time quantitative RT-PCR. *Br J Cancer* 1999;**80**:883–91.
- 52 Brakenhoff RH, Stroomer JG, ten Brink C, *et al*. Sensitive detection of squamous cells in bone marrow and blood of head and neck cancer patients by E48 reverse transcriptase-polymerase chain reaction. *Clin Cancer Res* 1999;**5**:725–32.
- 53 Melo JV, Yan XH, Diamond J, *et al*. Reverse transcription/polymerase chain reaction (RT/PCR) amplification of very small numbers of transcripts: the risk in misinterpreting negative results. *Leukemia* 1996;**10**:1217–21.



Limitations of cytokeratin 20 RT-PCR to detect disseminated tumour cells in blood and bone marrow of patients with colorectal cancer: expression in controls and downregulation in tumour tissue

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