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


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. Unfortunately, a large number of patients with early stage colorectal cancer, who have a localised tumour at diagnosis, will develop recurrent or metastatic disease, 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. 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.

“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. In colorectal cancer, cytokeratin 20 (CK20) is considered a useful marker, although its suitability is disputed. Some studies report a relation between CK20 mRNA detection in blood or bone marrow and stage of disease or prognosis. 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 or CK20 mRNA was almost solely identified in samples of patients with extensive disease or in no patient samples at all. Moreover, little is known about CK20 mRNA expression in colorectal tumours. Moll and colleagues 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.

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 patients with colorectal cancer. 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

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 classification13: 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 colonic 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 precipitated 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’-CTGGTAACGCGCATGC GGCT-3’ (sense) and 5’-GCAGATGCCCTCGATGGTGA-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 colleagues14, namely: 5’-CAGACACAGGGTG AACTATGG-3’ (sense) and 5’-GATCAGCTCCACGTTAGA 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, 200µM 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. Every experiment with a selected amount of cDNA 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 semi-quantified 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 K, 20.8
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
<table>
<thead>
<tr>
<th>Number of positive tests out of four CK20 PCRs/sample</th>
<th>Positive/total patients</th>
</tr>
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<tbody>
<tr>
<td>Blood</td>
<td>0 1 2 3 4</td>
</tr>
<tr>
<td>Healthy donor</td>
<td>13 3 0 0 0</td>
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<tr>
<td>Non-cancer colon patient</td>
<td>9 4 0 0 0</td>
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<tr>
<td>Non-cancer/non-colon patient</td>
<td>15 4 2 1 0 0</td>
</tr>
<tr>
<td>Bone marrow</td>
<td>11 4 0 0 0</td>
</tr>
<tr>
<td>Non-cancer colon patient</td>
<td>1 1 0 0 0</td>
</tr>
<tr>
<td>Non-cancer/non-colon patient</td>
<td>10 3 0 0 0</td>
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| Table 2 | Cytokeratin 20 (CK20) PCR of blood samples of patients with colorectal cancer
<table>
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<tr>
<th>Number of positive tests out of four CK20 PCRs/sample</th>
<th>Positive/total patients</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stage</td>
<td>0 1 2 3 4</td>
</tr>
<tr>
<td>Stage I</td>
<td>10 3 2 0 0</td>
</tr>
<tr>
<td>Stage III</td>
<td>7 3 0 0 0</td>
</tr>
<tr>
<td>Stage IV</td>
<td>4 0 0 1 0</td>
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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.
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, Ks 20.8 and Ks 20.10. The staining pattern for both antibodies was identical. However, staining with Ks 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. 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. 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>
<thead>
<tr>
<th>Table 3</th>
<th>Cytokeratin 20 (CK20) PCR of bone marrow samples of patients with colorectal cancer</th>
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<tr>
<td>Number of positive tests out of four CK20 PCRs/sample</td>
<td>Positive/total patients</td>
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<tr>
<td>Stage II</td>
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<td>Stage III</td>
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<td>Stage IV</td>
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<th>Table 4</th>
<th>Cytokeratin 20 (CK20) amplification in blood and bone marrow of the same patient with colorectal cancer</th>
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<tr>
<td>Bone marrow</td>
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<td>Blood</td>
<td>Positive</td>
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<th>Table 5</th>
<th>Cytokeratin 20 (CK20) mRNA and protein expression, measured by means of immunohistochemical staining with antibody Ks 20.10, in normal colon samples</th>
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<tr>
<td>No.</td>
<td>Epithelial cells (%)</td>
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<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>40</td>
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<td>2</td>
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<td>3</td>
<td>25</td>
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<td>40</td>
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<td>13</td>
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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.
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. Unfortu-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.11 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 peroperative bone marrow sample remained disease free after a follow up period of 19 months after surgery. Of the 20 patients who had
CK20 RT-PCR in colorectal cancer

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.28 29 35 46 However, it does not explain our results or the results of Champlevoyer and colleagues47 or Little and co-workers48 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 master genes carcinoembryonic antigen and CK19 in vitro and in vivo.49 However, CK20 mRNA expression in haematopoietic cells was not influenced by cytokines or growth factors when tested in the same in vitro test system.50 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.51 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.52 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 41–45 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.53–56 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.57–62 Obvious differences in methods are the selection of the PCR primers,63 the number of PCR cycles,64 the number of PCR reactions performed for each sample, and the interpretation of conflicting PCR results.63–65 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 24–46 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,40–45 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.7 Regarding CK20 protein expression, Moll and colleagues46 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.7 Another study showed that the CK20 protein is expressed, but that the mRNA is downregulated in colorectal tumour tissue compared with normal colon mucosa.47 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 disseminated tumour cells present in the blood and bone marrow of tumour bearing patients, needs to be examined.

ACKNOWLEDGEMENTS

The authors thank the Nijbakker-Morra foundation for their financial support.

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