Critical Evaluation of Real-Time Reverse Transcriptase-Polymerase Chain Reaction for the Quantitative Detection of Cytokeratin 20 mRNA in Colorectal Cancer Patients

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We evaluated the usefulness of cytokeratin 20 (CK20) mRNA expression in the quantitative detection of circulating tumor cells in the blood of patients with colorectal cancer (CRC). Blood samples from healthy volunteers (HVs; $n = 37$), patients with localized ($n = 42$) and metastatic colorectal cancer ($n = 40$), and patients with chronic inflammatory bowel disease (CID; $n = 15$) were examined. After immunomagnetic enrichment using microbeads against human epithelial antigen, total RNA was extracted, reverse transcribed, and analyzed by real-time reverse transcriptase-polymerase chain reaction using the LightCycler instrument. CK20 expression in peripheral blood was found in 46 of 82 (56%) patients with CRC, 8 of 37 (22%) HVs, and 9 of 15 (60%) patients with CID. Levels of CK20 mRNA were significantly higher in blood samples from CRC patients (median 681) than in blood samples from HVs (median 0) ($P = 0.001$), whereas no difference could be detected between patients with CRC and CID. Although the present technique could not distinguish CRC from CID, the method warrants further efforts to improve sample preparation and tumor cell enrichment, which may render real-time CK20 reverse transcriptase-polymerase chain reaction a feasible technique in identifying circulating tumor cells in peripheral blood of cancer patients. (*J Mol Diagn* 2005, 7:631–637)

The most important factor influencing the outcome of patients with invasive cancer is whether the tumor has spread, either regionally or systemically. Despite a primary curative treatment, however, a proportion of patients with no clinical evidence of systemic dissemination will develop recurrent or metastatic disease.^{1,2} It is assumed that disease progression often occurs due to the

presence of micrometastases that are currently undetectable by conventional methods. Numerous studies have analyzed the presence and significance of circulating tumor cells (CTCs) in peripheral blood, bone marrow (BM), and lymph nodes.^{2–7} To date, sufficient data are available from several large trials which demonstrate the poor prognostic influence of disseminated tumor cells present in BM on outcome in breast cancer patients.^{8–10} Two recently published studies clearly showed that in metastatic breast cancer, CTCs can predict progression-free and overall survival as well as response to treatment.^{11,12} Therefore, there is great interest in improved methods for the detection of CTCs in patients with solid tumors.

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 CTCs. For these purposes, many genes are being evaluated for their suitability, with the prime candidates being those with restricted expression. Cytokeratin 20 (CK20) belongs to the epithelial subgroup of the intermediate filament protein family that is involved in cell structure and differentiation. Expression studies showed that CK20 is restricted to gastrointestinal epithelium, urothelium, and Merkel cells of the skin^{13,14} and that this profile is maintained in malignant tumors of these cells.¹⁵ Furthermore, no processed CK20 pseudogenes have been found.¹⁴ Promising results have been published showing that the presence of CK20 expression in blood and BM samples from patients with colorectal cancer (CRC) is correlated with advanced stage of disease^{16–18} and shorter survival.^{19,20}

Whereas the sensitivity of RT-PCR assays is very high, ie, one tumor cell in 10^6 to 10^7 mononucleated blood cells (MNCs), enhanced detection is associated with a high false-positive rate. Especially RT-PCR studies using epithelial cell markers lack the prerequisite specificity to detect rare tumor cells in blood.^{18,21–23} The high false-

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positive rates seem to arise from ectopic transcription of epithelial markers.²⁴ Therefore, immunomagnetic or density gradient tumor cell enrichment has been used in several studies to improve both sensitivity and specificity by eliminating nucleated blood cells.^{16,24–26} Nevertheless, conflicting results have been published concerning the specific detection of CK20 in peripheral blood and comparison of sensitivities and specificities across different studies is difficult because the methods of sample preparation and experimental conditions vary.^{27,28}

The lack of a standardized method to detect disseminated tumor cells is a clear barrier to the clinical implementation of CTC detection. As a result, there is an important need to continue further studies on CK20 to involve standardized techniques with an ability to discriminate expression levels in a quantitative RT-PCR experiment.

The recent availability of real-time PCR enables the quantification of low-level background transcription and allows the definition of cutoff values for marker expression in blood and thus improves specificity.^{29–32}

In this study, we selected a quantitative real-time RT-PCR (qRT-PCR) based on a commercially available kit, because this approach offers the potential for improved standardization and quantification.^{28,33} Furthermore, the reliable quantification of housekeeping gene expression allows an excellent quality control on a per-sample basis and relates marker concentration to sample quality. To improve specificity and sensitivity, specimens were subjected to an immunomagnetic enrichment method before qRT-PCR analysis. To elucidate the usefulness of this CK20 qRT-PCR in combination with human epithelial antigen (HEA) microbead enrichment, we prospectively collected blood from a series of patients with localized and metastatic colorectal cancer. The specificity of the CK20 qRT-PCR was studied in two groups of noncancer controls: healthy volunteers, and patients with chronic inflammatory bowel disease.

Materials and Methods

Cell Lines and Spiking Experiments

HT29 colorectal cells were used for the spiking experiments. The HT29 cells were maintained in Dulbecco's modified Eagle's medium containing 10% fetal calf serum. HT29 tumor cells (1×10^7) were suspended in 1 ml of phosphate-buffered saline (PBS) and serially diluted 10-fold into 5×10^6 MNCs prepared from 20 ml of blood from a healthy volunteer. The final concentration of tumor cells was 10^6 , 10^5 , 10^4 , 10^3 , 10^2 , 10^1 , and 10^0 .

Patients and Controls

A total of 82 patients, aged 32–83 years (median 63), with histologically confirmed CRC (40 rectal and 42 colonic) were included in this study. Blood samples were collected from 40 patients with localized colorectal cancer (LCC) and from 42 patients with metastatic colorectal cancer (MCC) during therapy. According to the Union

International Contre le Cancer guidelines,³⁴ 2 (2.4%) of the carcinomas were stage I; 11 (13.4%), stage II; 26 (31.7%), stage III; and 43 (52.4%), stage IV. Negative controls were blood samples from healthy volunteers (HVs; $n = 37$) and from patients with inflammatory bowel disease (CID; $n = 15$).

Blood Sample Collection

Blood samples (10 ml) were collected in EDTA Vacutainer tubes (Becton Dickinson, Plymouth, UK) and stored at 4°C for a maximum of 2 hours before the experiments. To avoid contamination with skin epithelial cells, second draws were used. Ethical approval for blood sample collection for this research was obtained from the local ethical committee. All patients had given written informed consent for the analysis.

Determination of Assay Specificity

Ten of 37 blood samples from HVs and five blood samples from patients with CID were analyzed to examine CK20 mRNA expression in different blood fractions. Therefore, blood samples were processed in the same way as the patients' specimens described below. Additionally, 10 ml of the sample were used for Ficoll plaque density gradient centrifugation according to the manufacturer's instructions (Lymphoprep; Axis Shield PoC, Oslo, Norway). MNCs at the interface were harvested and washed with PBS. Then cells were lysed in 400 μ l of lysis buffer (Roche Diagnostics, Vienna, Austria) and stored at -20°C until RNA extraction. Additionally, the layer containing the polymorphonuclear (granulocyte) fraction was further processed by osmotic erythrocyte lysis and subsequent wash steps to remove inhibitory hemoglobin from the cell fraction before CK20 qRT-PCR amplification.

Enrichment

Immunomagnetic cell selection using an anti-HEA-125 magnetic microbead conjugate was performed for labeling and separation of epithelial cells (Miltenyi Biotec, Bergisch Gladbach, Germany). The system and the magnetic cell separation column were used according to the manufacturer's instructions. Briefly, after red blood lysis, cells were washed and incubated with HEA-conjugated microbeads. After incubation, cells were washed and resuspended in 2 ml of PBS with 0.1 mmol/L EDTA and subsequently applied to the MACS CS+ column. Positively enriched cells (in 2 ml of PBS with 0.1 mmol/L EDTA) were transferred into a sterile Eppendorf tube, washed in PBS, and resuspended in 200 μ l of PBS. After addition of 400 μ l of lysis buffer (Roche Diagnostics), cells were stored at -20°C until RNA extraction.

RNA Extraction

Total RNA was extracted from enriched cells using the commercially available High Pure RNA Isolation kit

(Roche Diagnostics) according to the manufacturer's instructions. All of the steps were performed using sterile technique in separate rooms with designated areas for RNA extraction, RT-PCR, and sample analysis to reduce the risk of contamination.

CK20 Real-Time RT-PCR

All reagents were from Roche Diagnostics and were included in the LightCycler CK20 mRNA Quantification kit. A detailed description of this assay has been published elsewhere.^{21,33} Briefly, cDNA was synthesized by reverse transcription using 10 μ l of RNA and random hexamers serving as primers in a total volume of 20 μ l in a thermal cycler (MyCycler; Bio-Rad, Munich, Germany). The samples were incubated at 25°C for 10 minutes, at 42°C for 30 minutes, and then at 94°C for 5 minutes. Samples were placed on ice until amplification by PCR.

Each reaction contained 2 μ l of the cDNA, 1 $\times$ DNA master hybridization probes mix, and 1 $\times$ detection mix in a 20- μ l volume. The detection mix includes forward and reverse primers, a pair of hybridization probes (LC-Red 640 and fluorescein), and 40 mmol/L MgCl₂. Using the same cDNA preparation but in a separate PCR, mRNA encoding for phosphobolinogen deaminase (PBGD) was processed and used as housekeeping gene. Its product served as the control for RNA and relative quantification. Cycling parameters were as follows: an initial denaturation at 95°C for 10 minutes; 10 seconds at 95°C and 60°C; and 5 seconds at 72°C for 50 cycles. The PCR run was concluded with a 40°C incubation for 30 seconds. For all RT-PCR steps, negative controls were performed, including a reverse transcriptase negative control, a no template control (water), and a negative sample control.

CK20 gene levels were determined using the efficiency-corrected calibrator normalized relative quantification by the LightCycler Relative Quantification Software 4.0. The crossing point was determined by the second derivative maximum method. In the CK20 kit, the ratio of target to reference is set to a value of 1,000,000 for easier reading of the results. Efficiency-corrected ratios of the calibrator were used as an inter-run quality control.

Statistical Analysis

A comparison of CK20 mRNA levels among HVs, colorectal patients, and patients with CID was performed using nonparametric Kruskal-Wallis test (SPSS version 13 for Windows; SPSS, Inc.) followed by post hoc Dunn's multiple comparison test (SigmaStat version 3.1; SPSS, Inc.), respectively. Differences were considered significant if $P < 0.05$.

Sensitivity and specificity of CK20 mRNA ratio values were determined based on findings in cancer patients ($n = 82$) and in the HV group ($n = 37$). The threshold value for optimal sensitivity and specificity of CK20 mRNA ratio was determined by receiver operating characteristics (ROC) curve. The cutoff value that maximized the sum of sensitivity and specificity for discrimination

Figure 1. Box plots show that expression of PBGD was not significantly different among HVs, patients with CRC (Pat), and patients with CID ($P = 0.100$; Kruskal-Wallis test).

between CRC patients and HVs was chosen for CK20 mRNA positivity.

Results

PBGD as an Endogenous Control

A comparison of PBGD expression levels between HVs, patients with CRC, and patients with CID was made using all available crossing point values for PBGD. There was no significant difference in the median CP values between the groups ($P = 0.100$; Figure 1).

The lack of significant variation in PBGD expression between HVs and diseased patients proved it suitable as an endogenous control for the relative quantification of CK20 mRNA in patients with CRC.

Determination of Assay Sensitivity

In cell spiking experiments in which decreasing numbers of HT29 cells were added to blood from a HV, CK20 qRT-PCR detected consistently 10^1 cells in a background of 5×10^6 leukocytes, and 10^0 cells gave inconsistent negative or positive results. The fitted regression line from these data exhibited an excellent linearity of $r^2 > 0.99$ (data not shown).

Specificity of the Assay

To examine the influence of CK20 mRNA expression from nucleated blood cells on qRT-PCR specificity, we investigated different blood fractions in a panel of peripheral blood samples from HVs ($n = 10$) and patients with CID ($n = 5$). In addition to HEA enrichment, blood samples were subjected to a standard Ficoll density gradient sep-

Figure 2. ROC curve for CK20 mRNA ratios to distinguish CRC patients ($n = 82$) from HVs ($n = 37$). ROC curves were constructed by plotting sensitivity and 100-specificity corresponding to each cutoff value for CK20 mRNA. The cutoff of 415 gave a sensitivity of 55% and a specificity of 78%. Area under the curve = 0.672; SE = 0.051; 95% confidence range, 0.580–0.756; $P < 0.003$.

aration of the mononuclear and granulocyte cell fraction. In both HEA-enriched cells and MNCs, 27% (4/15) of the samples were positive for CK20 mRNA. As expected, CK20 expression was identified in the majority of the granulocyte fraction (87%).

ROC Curve Analysis

The area under the ROC curve for CK20 mRNA ratios was 0.672 (confidence range, 0.580 to 0.756; $P < 0.003$). Optimal sensitivity (55%) and specificity (78%) was obtained by applying 415 as a CK20 mRNA ratio cutoff value (Figure 2). This cutoff was used to define positive samples in all of the three groups analyzed.

Healthy Volunteers and Patients with Inflammatory Bowel Disease

Using >0 as a cutoff threshold, 10 of 37 (27%) HVs and 9 of 15 (60%) CID patients were positive for CK20 mRNA, with a median ratio of 0 (range, 0 to 2680) and 940 (range, 0 to 4880), respectively. Using the cutoff ratio of

Figure 3. Comparison of CK20 mRNA ratio between patients with CRC, LCC, and MCC; HVs; and patients with CID. **Black bars**, median; Cutoff value 415 is indicated by **dashed horizontal line**.

>415 , 8 of 37 (22%) blood samples from HVs and 9 of 15 (60%) CID patients were positive (Table 1).

Colorectal Cancer Patients

Using >0 as a cutoff threshold, 46 of 82 (56%) blood samples from patients were positive for CK20 mRNA. The median CK20 mRNA ratio was 681 (range, 0 to 19,700). In the group of patients with LCC, 23 of 40 (58%) were positive, with a median CK20 mRNA ratio of 665 (range, 0 to 11,700). CK20 mRNA expression was found in 23 of 42 patients with MCC (55%), with a median of 681 (range, 0 to 19,700). Using the >415 threshold, 45 of 82 (55%) CRC patients, 23 of 42 (55%) MCC, and 22 of 40 (55%) LCC patients were positive for CK20 mRNA (Table 1).

There was a significant difference in the relative CK20 mRNA levels among HVs (median, 0), patients with CRC (median, 681), and patients with CID (median, 940) ($P = 0.002$; Kruskal-Wallis test; Figure 3). Posthoc Dunn's multiple comparison test revealed significantly higher ratio levels of CK20 mRNA in blood samples from patients compared with HVs ($P = 0.001$). No difference in ratio levels was found between blood samples from patients with LCC and MCC ($P = 0.831$). Similarly, there was no significant difference between patients with CRC and patients with CID ($P = 0.797$). Figure 4 illustrates a representative example of a CK20 qRT-PCR run.

Table 1. Detection of CTCs by CK20 qRT-PCR in Blood Samples from HVs, Patients (pt) with CID, and Patients with CRC, LCC, or MCC

CK20 Ratio	HV	CID	All CRC	LCC	MCC
<i>n</i>	37	15	82	40	42
Median	0	940	681	665	681
Minimum	0	0	0	0	0
Maximum	2,680	4,880	19,700	11,700	19,700
Number of pt >0	10 (27%)	9 (60%)	46 (56%)	23 (58%)	23 (55%)
Number of pt $>$ cut-off 415	8 (22%)	9 (60%)	45 (55%)	22 (55%)	23 (55%)

Figure 4. Representative results of a CK20 qRT-PCR using the LightCycler. **Solid lines** indicate amplification curves from the target gene (CK20) and **dashed lines** from the reference gene (PBGD). No product was amplified in the no-template sample or when reverse transcription was omitted (●).

Discussion

In our study, we evaluated the frequency of CK20 mRNA expression in patients with LCC and MCC, HVs, and patients with CID using the qRT-PCR on the LightCycler. Immunomagnetic HEA microbeads were used to harvest epithelial cells from peripheral blood using a semiautomated system (Automacs). We selected a HEA antibody directed against the epithelial cell adhesion molecule, because this cell surface protein is expressed in epithelial tissues and most neoplasms derived from these tissue.³⁵ Although HEA is usually expressed on the epithelial cell surface, its expression is not constant and varies among specimens;^{36–38} this represents one limitation of this enrichment technique. CRC cases that lack epithelial antigen are missed by this enrichment method, and this could contribute to the low sensitivity. Based on studies from model systems, the currently used enrichment techniques are all associated with loss of target cells to varying degrees and the qRT-PCR signal level may be influenced because of this loss. Despite this fact, the literature suggests the urgent need of sample enrichment before RNA extraction for quantitative PCR analysis to enhance the specific signal from target tumor cells.

In the literature, different frequencies for CK20-positive patients have been described.^{16,17,39,40} Differences in methods of RT-PCR and sample preparations may account for these conflicting results. Consequently, reasonable comparison between the studies is difficult, and standardization is urgently needed.²⁸

Using a cutoff ratio of 415, we found 55% of the patients with CRC, 22% HVs, and 60% CID patients positive for CK20 mRNA. The percentage of positive cases was

the same in LCC and MCC patients (55%). CK20 expression was not significantly higher in patients with MCC compared with those with LCC ($P = 0.831$). A possible explanation for this finding might be the fact that MCC patients received chemotherapy at the time of blood draw in contrast to no treatment in LCC patients. It has been published previously that response to treatment in the metastatic setting has an influence on the detection rate of CTCs in peripheral blood.^{12,41} The fact that MCC patients received chemotherapy might also contribute to the higher median of CID patients compared with the median of CRC patients. Although the inclusion of MCC patients who are not on chemotherapy might have contributed to a higher positivity rate, the problem of low specificity would still remain.

These results and the high number of positive cases in the control groups were unexpected, particularly because an immunomagnetic enrichment technique was performed for all samples before PCR analysis. As a consequence, we studied CK20 mRNA expression in two different white blood cell fraction separated by gradient centrifugation: mononuclear cells and granulocytes. We detected a high frequency of CK20 mRNA expression in normal granulocytes (87%), and low-level expression in both mononuclear cells and HEA-enriched cell fraction (27% each). These results confirm the findings by others^{27,42} who also reported a remarkable CK20 mRNA expression in the granulocyte fraction but also in enriched tumor cells by immunomagnetic separation and density-gradient enrichment. Thus, modification and improvement of the enrichment step is still an important issue to further progress on the qRT-PCR results.

The results in the literature regarding the detection of CK20 mRNA in blood and bone marrow of noncancer controls remain controversial. Illegitimate transcription of CK20 and other tissue-specific genes in blood from HVs has been previously described by the mononuclear fraction.^{21,29,43,44} Schuster et al³⁹ question the suitability of peripheral blood as the compartment for detection of CTCs in colorectal cancer because they were not able to differentiate satisfyingly mRNA markers originating from tumor cells and those from illegitimate transcription in hematopoietic cells in blood. However, it is important to mention that they did not use any tumor cell enrichment before qRT-PCR. Illegitimate transcription of cytokeratins in blood cells is a well-known limitation and lowers the specificity of the RT-PCR analysis. Other studies, however, showed no CK20 expression in the blood of non-cancer controls.^{17,23,45–47}

In our study, background mRNA expression in CID patients and HVs could not be avoided, although specimens were subjected to HEA enrichment before qRT-PCR analysis. These results confirm the findings of others who demonstrated that hematopoietic cells may be non-specifically retained during immunomagnetic enrichment of epithelial cells.^{31,48} One group²⁶ showed that only a combination of both negative and positive immunomagnetic isolation revealed the highest sensitivity and specificity for detecting CTCs in colorectal cancer. Neverthe-

less, the clinical applicability must be questioned when such a complex technique is used. Another study⁴³ reported that the CD34⁺ fraction of mononuclear cells is a source of ectopically expressed epithelial cell-specific markers and that these CD34⁺ cells may contribute to the high false-positive rate observed when such markers are used to detect rare circulating metastatic cancer cells by RT-PCR. This could be another explanation for the high false-positive rates.

Our results also showed that CK20 is detected more frequently in blood samples of patients with CID compared with HVs. These results suggest that nonmalignant colonic epithelial cells may be shed into the bloodstream in the presence of bowel pathology.¹⁸ Furthermore, one has to keep in mind that CK20 is a tissue-specific marker but not a tumor-specific one, thus not able to discriminate between cancer cells and nonmalignant epithelial cells. In our study, no difference was found between CK20 expression levels in CRC patients and patients with CID. Normal colonic mucosal cells may also be circulating in CRC patients and thus cannot be distinguished from CTCs, making an interpretation difficult. Although CK immunohistochemistry could resolve the issue of tumor cell morphology and corroborate qRT-PCR data in true positive cases, this technique has an inherent limitation in its low sensitivity. Multimarker assays might overcome some of the inherent problems associated with single-marker techniques such as tumor heterogeneity and lack of specificity.⁴⁹ However, our results also emphasize the fact that in terms of specificity, it is not sufficient to evaluate the expression of the investigated tumor marker in HVs only, but also in a nonmalignant control group.

The technically demanding process of sample preparation should not be underestimated when using a seemingly easy and sensitive method as qRT-PCR. Variability in technical factors, such as different enrichment techniques, varying cell numbers, RNA isolation, and cDNA synthesis method, may influence the sensitivity and specificity of the detection methods, making a comparison across different studies problematical.^{5,27,28,42,50} We believe that the present CK20 mRNA qRT-PCR assay provides a useful standardized technique to detect CTCs, which can be perfected by improvement in pre-analytical parameters.

In conclusion, our results confirm that background transcription of CK20 by peripheral blood leukocytes cannot be avoided by immunomagnetic separation of epithelial cells using HEA microbeads. Although the present results show a clear limitation of a current clinical implementation of this technique, we believe that they warrant extended studies in this field. Further improvements and standardization of sample preparation and tumor cell enrichment may render CK20 qRT-PCR a feasible technique in detecting CTCs in peripheral blood of cancer patients. Identification of other target genes encoding for tumor-associated or tumor-specific antigens could also improve sensitivity and specificity, and in this context, the research for appropriate target genes remains an important issue.

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