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Specific Detection of Cytokeratin 20-Positive Cells in Blood of Colorectal and Breast Cancer Patients by a High Sensitivity Real-Time Reverse Transcriptase-Polymerase Chain Reaction Method

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A real-time reverse transcriptase-polymerase chain reaction (RT-PCR) method for detection of *cytokeratin 20*-positive cells in blood characterized by two novel features was developed and tested on 99 patients with colorectal cancer, 110 with breast cancer, and 150 healthy subjects. To optimize the specificity and sensitivity of the method, two novel features were used. First, a primer overlapping two adjacent exons was generated to inhibit nonspecific amplification both in healthy donors and cancer patients; second, a non-end-point first-round amplification was used to increase sensitivity. The number of first-round cycles was chosen to reach the highest level of sensitivity while conserving quantitative characteristics. PCR efficiency increased from 88.9% in single-round RT-PCR to 99.0% in nested real-time RT-PCR. To establish sensitivity and specificity of the method, HT29 cells were serially diluted with normal blood. Detection limit improved from 100 HT29 cells (single-round RT-PCR) to 1 to 10 cells (nested real-time RT-PCR) per 3 ml of whole blood. None of the healthy subjects was positive, whereas 22 and 29% of all colorectal and breast cancer patients, respectively, had *cytokeratin 20* cell equivalents in blood. The association between *cytokeratin 20* cell equivalents and metastasis was statistically significant for breast ($P = 0.026$) but not colorectal cancer patients ($P = 0.361$). Negativity of all 150 healthy controls examined confers diagnostic potential to the method. *(J Mol Diagn 2006, 8:105–112; DOI: 10.2353/jmoldx.2006.050054)*

Cytokeratin mRNAs are potential markers for detection of epithelial cells in blood. Several reports indicate that *cytokeratin 20* (*CK20*) mRNA in blood acts as a specific cancer cell marker in patients with frequent cancer forms of epithelial origin such as breast[1–5] and colorectal cancer.[6–9] Many breast cancer patients develop metastasis after locoregional and systemic treatment even in the absence of dissemination as assessed by conventional diagnostic tools. Approximately 30 to 50% of colorectal cancer patients who have undergone curative resection have recurrences with fatal outcome.[10,11] Most recurrences occur in patients with TNM (tumor, nodes, and metastases) stage II and III cancers, but patients with stage I lesions also have appreciable risk. In these patients, cancer cells were disseminated either before or during surgery of the primary tumor.[12–14] Although the relationship between circulating tumor cells and the development of recurrent cancer is not fully understood, it is generally assumed that enhanced dissemination of cancer cells in blood contributes significantly to the development of metastasis.[15–17] The detection of circulating metastatic cells would be of great value for the assessment of the metastatic risk.[18] Currently, the most powerful prognostic information in cancer patients is obtained from conventional histological assessment of regional lymph nodes.[19–23] Because 20 to 30% of colorectal cancer patients without metastasis in lymph nodes die from distant metastases or local recurrence within 5 years[24] and 15% of "node-negative" breast cancer patients will probably develop metastasis,[25] there is a strong need for specific and sensitive methods capable of detecting circulating cancer cells. The assay of tumor-specific mRNA

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Table 1. Primer Pairs Amplicons Analyzed by Real-Time PCR for Detection of *CK20* Gene (GenBank accession no. NM_019010)

	Sequence of selected primer pairs*		Length of amplicon (bp)	Predicted T_m of amplicon	Measured T_m of amplicon
Primer A	5′-TCTTTGATGACCTAACCCTACA-3′	Exon 3			
Primer B	5′-ATTGACAGTGTTGCCCAGAT-3′	Exon 4	Primers A and B: 137	83.4	85.5
Taqman probe	5′-CAGGAGGAAGTCGATGGCCTACAC-3′	Exon 3/4			
Primer C	5′-CAGACACACGGTGAACTATGG-3′	Exon 1			
Primer D	5′-GATCAGCTTCCACTGTTAGACG-3′	Exon 3	Primers C and D: 371		
Primer E	5′-GCAAATCAAGCAGTGGTACGAAAC-3′	Exon 1	Primers E and F: 110	85.7	87
Primer F	5′-GCAGTTGAGCATCCTTAATCTGAC-3′	Exon 1/2	Exon overlapping		
Primer G	5′-AATTTGCAGGACACACCGAGCA-3′	Exon 2	Primers E and G: 136	87	88

*Primers A, C, and E are forward; primers B, D, F, and G are reverse.

by quantitative reverse transcriptase-polymerase chain reaction (RT-PCR) appears to be the most sensitive method to identify and detect minimal numbers of cancer cells in peripheral blood,[6,26–30] but the interpretation of results is often difficult because of a variable number of positive normal subjects. The present available data do not allow determination of whether *CK20*-positive (*CK20*$^+$) cells found in blood of normal subjects are due to *CK20* expression of few normal epithelial cells released in the circulation or due to technical artifacts such as DNA co-amplification. Moreover, current methods are often not quantitative,[1,31] have low sensitivity,[6,31] possess high risk of cross-contamination, and have low specificity.[2,7,32–35] Very few reports compared results obtained in different cancer types by the same methodology[36] to ascertain whether differences were due to the cancer type or to the method used.

Aiming to increase sensitivity and abrogate false-positive results, we have developed a real-time RT-PCR method for detection of *CK20* cell equivalents in blood provided with two novel features: a primer overlapping two adjacent exons to inhibit nonspecific amplifications and a non-end-point first-round amplification to increase sensitivity. The method was tested in healthy subjects and in breast and colorectal cancer patients at different stages of tumor development. None of the 150 healthy subjects tested positive, whereas 22% of the 99 patients with colorectal cancer and 29% of the 110 patients with breast cancer had *CK20* cell equivalents in their blood. The association between *CK20* cell equivalents and metastasis was statistically significant for breast but not colorectal cancer patients.

Materials and Methods

cDNA Preparation from Venous Blood and Tumor Cell Line HT29 and MCF-7

Venous blood samples were obtained from 99 patients with colorectal cancer and 110 patients with breast cancer at different stages of tumor development. No particular effort was made to stratify the participants into well-defined groups with respect to stage because this was not the aim of this study. All patients had given informed consent for the study. One hundred fifty (74 males and 76 females) healthy volunteers (from Associazione Volontari Italiani del Sangue Blood Bank, Torino, Italy) aged 30 to 60 years served as controls. All patients and controls were not affected by reactive/inflammatory condition. The first 5 ml of blood were discarded to avoid contamination with skin cells.[37] The next 10 ml of blood were collected in vacutainer tubes with sodium citrate and used exclusively within 3 hours. Blood samples were diluted 1:3 in RPMI 1640 (Invitrogen, Carlsbad, CA), and mononuclear cells were separated using Ficoll density separation (Amersham Biosciences, Uppsala, Sweden). Tumor cell line HT29 (human colon adenocarcinoma) (Istituto Zooprofilattico B. Umbertini, Brescia, Italy) was maintained in continuous culture in McCoy's media (Invitrogen) containing 10% fetal bovine serum and antibiotics at 37°C with 5% CO_2. Tumor cell line MCF-7 (human breast adenocarcinoma) purchased from DSMZ (Braunschweig, Germany) was maintained in continuous culture in RPMI 1640 (Invitrogen) containing 10% fetal bovine serum and antibiotics at 37°C with 5% CO_2. RNA was extracted from 1×10^6 HT29 or MCF-7 cells and 3×10^6 mononuclear cells from healthy donors and patients by phenol-chloroform precipitation and microparticle RNA capture (Nurex, Sassari, Italy). cDNA was prepared by adding 30 μl of extracted RNA to 48 μl of reaction mixture containing 300 ng of oligo-dT and 600 U of Moloney-murine leukemia virus reverse transcriptase (both Invitrogen).

Primers and Standard

Different primer pairs (Invitrogen) and one Taqman probe were used for *CK20* PCR amplification (Table 1). The Taqman probe was labeled on the 5′ end with 6-carboxyfluorescein as the reporter and on the 3′ end with 6-carboxytetramethylrhodamine as the quenching dye. Oligonucleotide sequences were identified using Beacon Designer Software (PREMIER Biosoft International, Palo Alto, CA) and designed to differentiate between cDNA- and DNA-derived PCR products. Serial dilutions of cDNA from known numbers of HT29 cells were used to generate a standard curve.

Real-Time RT-PCR Using Taqman Probe or SYBR-Green and HT29 Cells

Real-time RT-PCR of *CK20* mRNA was performed on the i*Cycler* instrument (Bio-Rad, Hercules, CA). *CK20*-specific primers A and B spanning 137-bp sequence and the

Taqman probe hybridizing to the target sequence were used. PCR amplification (50 cycles, 94°C, 30 seconds; 58°C, 30 seconds; and 72°C, 30 seconds) was performed in 50 μl of reaction mixture using serial dilutions of HT29 cells cDNA. Ten μL of template cDNA was added to the amplification mixture containing 200 nmol/L primer pair A and B, 2.5 U of Platinum *Taq*DNA Polymerase (Invitrogen), 3 mmol/L $MgCl_2$, 200 μmol/L dNTP mixture (Applied Biosystems, Foster City, CA), and 200 nmol/L probe or 3.25 μl of SYBR-Green (Sigma-Aldrich, St. Louis, MO) diluted 1:10,000. DNA polymerase was pre-activated for 2 minutes at 94°C.

Single-Round Real-Time RT-PCR (S-PCR) Using SYBR-Green and HT29 Cells

S-PCR (50 cycles, 94°C, 30 seconds; 64°C, 30 seconds; and 72°C, 30 seconds) was performed in 50 μl of reaction mixture using serial dilutions of HT29 cells cDNA. Ten μL of template cDNA was added to the amplification mixture containing 200 nmol/L primer pair E and F, 2.5 U of Platinum *Taq*DNA Polymerase, 3 mmol/L $MgCl_2$, 200 μmol/L dNTP mixture, and 3.25 μl of SYBR-Green diluted 1:10,000. DNA polymerase was pre-activated for 2 minutes at 94°C.

Nested Real-Time RT-PCR (N-PCR) Using SYBR-Green on HT29 Cells and Clinical Samples

N-PCR first-round amplification (20 or 35 cycles, 94°C, 30 seconds; 65°C, 30 seconds; and 72°C, 30 seconds) was performed in 100 μl of reaction mixture. Twenty microliters of cDNA obtained from mononuclear cells were added to the amplification mixture containing 100 nmol/L primer pair C and D,[1] 5 U of Platinum *Taq*DNA Polymerase, 3 mmol/L $MgCl_2$, and 200 μmol/L dNTP mixture. One microliter of this mixture was re-amplified (35 cycles, 94°C, 30 seconds; 64°C, 30 seconds; and 72°C, 30 seconds) in 50 μl of reaction mixture using 200 nmol/L primer pair E and G or E and F, 2.5 U of Platinum *Taq*DNA Polymerase, 3 mmol/L $MgCl_2$, 200 μmol/L dNTP mixture, and 3.25 μl of SYBR-Green diluted 1:10,000. DNA polymerase was pre-activated for 2 minutes at 94°C. A standard curve with four dilutions of HT29 cells cDNA was included in each respective PCR run to quantify the number of *CK20* cell equivalents in clinical samples. All samples were analyzed in triplicate. To avoid contaminations, precautions included separate rooms and laboratory accessories for blood sampling, RNA isolation, PCR first round, and PCR second round.

Spiking Experiments and Cell Treatments

Varying numbers of HT29 cells (1 to 10^4 cells) or MCF-7 cells were added to 3 ml of blood and separated with mononuclear cells using Ficoll density separation. Alternatively, epithelial cells were separated from blood by Epithelial Enrich Ber-EP4-coated microsize immunobeads (Dynal Biotech, Success, NY) as described in the data sheet. RNA was purified as described above or in combination with a DNase treatment (Ambion, Austin, TX). RNA was also extracted and purified from cells after the removal of nuclei by treating cells with a 50 mmol/L Tris buffer, pH 8.0, containing 140 mmol/L NaCl, 1.5 mmol/L $MgCl_2$, 1 mmol/L dithiothreitol, 0.5% Igepal CA-630 (Sigma-Aldrich), and Recombinant Ribonuclease Inhibitor (Invitrogen). Cells were incubated on ice for 5 minutes, lysates were centrifuged at 4°C for 2 to 3 minutes at 300 × *g*, and supernatants were collected for RNA extraction.

Sequence Analysis

Direct sequencing of PCR products from HT29 cells and *CK20*$^+$ cells of colorectal cancer patient was performed on the ABI Prism 310 DNA sequencer (Applied Biosystems) using primers E and F (Table 1). Sequencing reactions were conducted with the big dye terminator sequencing ready reaction kit (Perkin-Elmer, Boston, MA).

Statistical Analysis

The association between metastasis and a detectable number of *CK20* cell equivalents in blood samples was tested in 99 colorectal and 110 breast cancer patients. The presence of *CK20* cell equivalents was analyzed as a dichotomous response variable (present/absent) in the two cancer groups, and the association with metastasis tested via the χ^2 test statistic. The probability distributions of the number of *CK20* cell equivalents in metastatic versus nonmetastatic patients were then compared through a nonparametric test (Wilcoxon-Mann-Whitney rank sum test) in both colorectal and breast cancer patients. Results were considered statistically significant for $a = 0.05$.

Results

Comparison between Taqman Probe and SYBR-Green Detection Methods Using HT29 Cells

To compare Taqman probe and SYBR-Green detection methods, cDNA from HT29 cells was used at different dilutions corresponding to a range between 1 and 10^5 cells. The detection limit of SYBR-Green detection method, expressed as minimal number of detectable cells, was at least 10-fold lower than that of the Taqman probe method (not shown).

Comparison between S-PCR and N-PCR Using HT29 Cells

Because of its higher sensitivity, SYBR-Green was used to monitor real-time RT-PCR reactions instead of Taqman probe. Confirmation of results by melting curve analysis

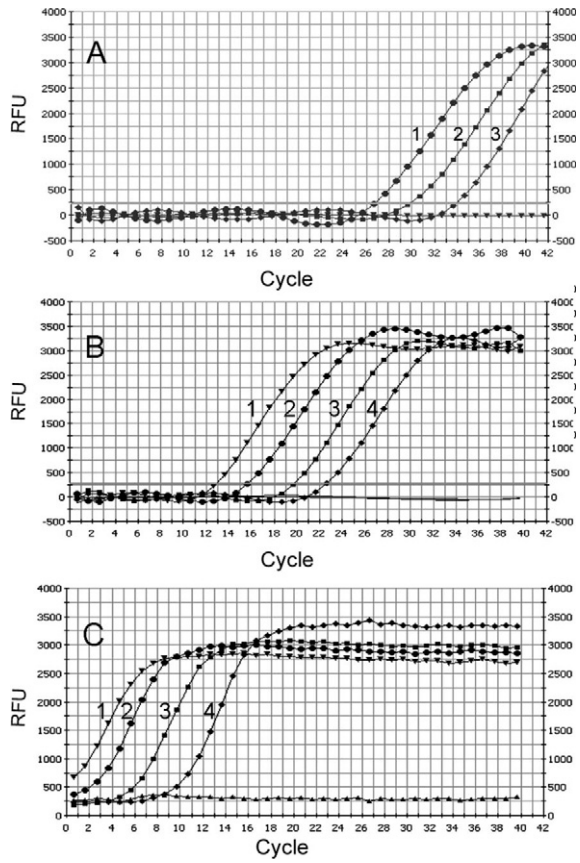

Figure 1. *CK20* amplification plots of serially diluted HT29 cells. Serially diluted HT29 cells (1, 10^4 cells; 2, 10^3 cells; 3, 10^2 cells; 4, 10^1 cells) amplified by S-PCR (**A**), 20N-PCR (**B**), and 35N-PCR (**C**). Numbers of cycles were plotted against fluorescence expressed as relative fluorescence units.

was an additional advantage conferred by SYBR-Green usage. S-PCR and N-PCR, with 20 cycles (20N-PCR) and 35 cycles (35N-PCR), were performed using serial dilutions of cDNA from HT29 cells. Detection limits of 20N-PCR and 35N-PCR were identical. The detection limits of 20N-PCR (Figure 1B) and 35N-PCR (Figure 1C) were at least 10-fold lower than the detection limit obtained by S-PCR (Figure 1A). Sensitivity data are shown in Table 2. 35N-PCR did not allow cell quantification because PCR curves were not comprised in the instrumental quantifiable range (Figure 1C). 20N-PCR showed 99.0 ± 3.8% efficiency (slope, −3.35 ± 0.09; intercept, 29.105 ± 0.97; correlation coefficient, 0.998 ± 0.0008) in eight independent test runs, whereas S-PCR efficiency was 88.8 ± 10% (slope, −3.65 ± 0.3; intercept, 39.22 ± 2.4; correlation coefficient, 0.994 ± 0.004) in six independent test runs. The specificity of the N-PCR method was tested on HT29 cells and on mononuclear cells obtained from healthy donors. Using HT29 cells, conventional intron-spanning primers (Table 1, primers E and G) often revealed two separate peaks by melting curve analysis, one corresponding to the expected melting temperature (T_m) and one corresponding to higher T_m (Figure 2A). This observation may indicate that the PCR reaction was influenced by the co-amplification of traces of residual DNA, as confirmed by experiments in which the reverse transcription step was performed in the absence of reverse transcriptase. Moreover, 20N-PCR analysis on lympho-monocytes isolated from healthy donors followed by 32 to 34 PCR cycles consistently revealed amplification signals (Figure 3A). In addition, melting curve analysis also revealed nonspecific amplifications (Figure 3B). The presence of nonspecific amplification products was also clearly documented by gel electrophoretic analysis (Figure 3C). To inhibit nonspecific amplifications, primer G was substituted by primer F designed to overlap exons 1 and 2 (Table 1; Figure 4) and containing only 5 bases on exon 1. Of note, when primer F contained more than 10 bases complementary to exon 1, some DNA amplification still occurred (not shown). Absence of DNA amplification using primer E in combination with primer F was confirmed by the absence of any amplification signal when the reverse transcription step was omitted (not shown).

In HT29 cells, a single peak, corresponding to the specific T_m, indicated absolute specificity of the combination between primers E and F (Figure 2B). Observed T_m was 87.3 ± 0.2°C in 14 independent test runs. Amplification specificity was further confirmed by sequencing the PCR products, which were found to be homologous with the *CK20* sequence from the National Center for Biotechnology Information database. The sensitivity obtained with primers E and F was identical to the sensitivity obtained with primers E and G. The analytical reproducibility of 20N-PCR with primers E and F was tested with varying HT29 cell numbers, and PCR assays were performed on the same cDNA in six triplicate experiments: means, SD, and variation coefficient (CV) are shown in Table 3. Melting curve analysis was also tested in all $CK20^+$ clinical samples (see below) and consistently showed one specific peak (Figure 2C).

Experiments Using HT29 or MCF-7 Cells Spiked in Blood

To mimic a diagnostic situation of metastatic cells in clinical samples, different dilutions of HT29 or MCF-7 cells were

Table 2. Detection Limit* of S-PCR and N-PCR

Sample	A. HT29	B. HT29/blood	C. HT29/blood	D. HT29/blood	E. HT29/blood
Treatments	–	–	Immunocapture	DNase treatment	mRNA enrichment
S-PCR	10 to 100	100	100	100	100
20N-PCR	1 to 10	1 to 10	10 to 100	1 to 10	1 to 10
35N-PCR	1 to 10	1 to 10	10 to 100	1 to 10	1 to 10

*Detection limit is expressed as the minimum number of cells detected in triplicate experiments.
A, HT29 cells; B, D, and E, HT29 cells added to 3 ml of blood and isolated by Ficoll density separation; C, HT29 cells isolated by immunocapture; D, DNase treatment; and E, mRNA enrichment by nuclei removal.

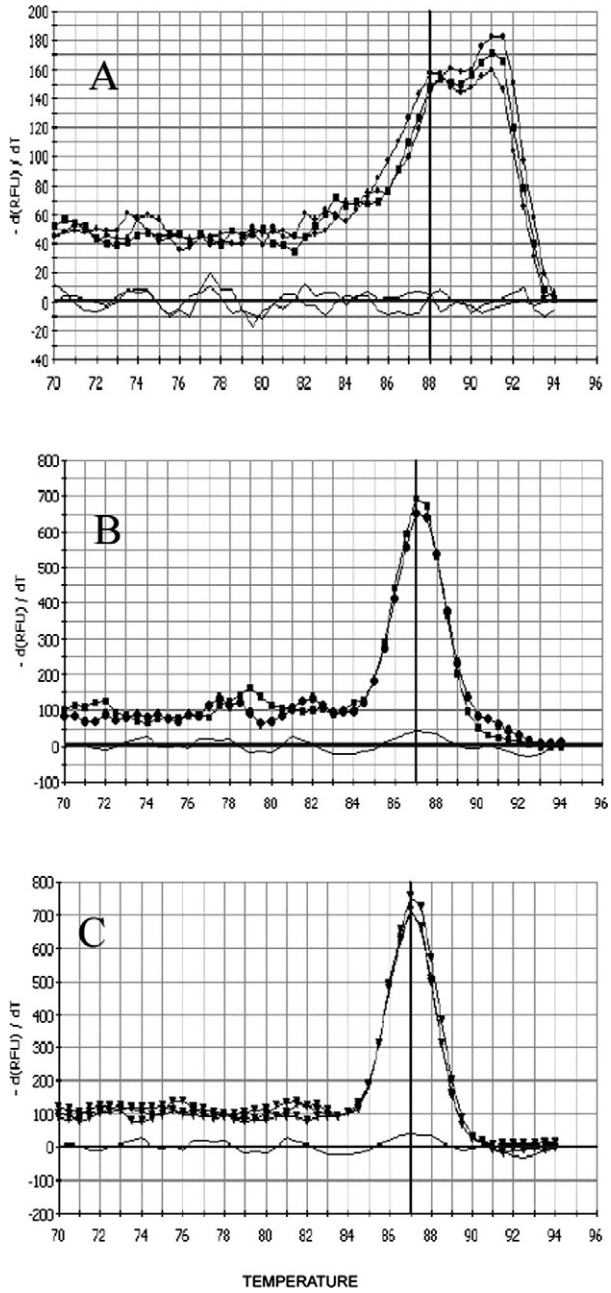

Figure 2. Melting peak analysis of 20N-PCR products. The melting peaks result from plotting the negative first derivative of measured fluorescence emission (*y* axis) at a given temperature (*x* axis). Curves were obtained with pure HT29 cells (**A** and **B**); with *CK20*$^+$ clinical samples (**C**); with intron-spanning primers E and G (**A**); and with primer E and exon-overlapping primer F (**B** and **C**).

spiked in 3 ml of blood obtained from healthy controls. The detection limit of 20N-PCR ranged between 1 and 10 HT29 or MCF-7 cells, indicating no interference due to the addition of a large excess of blood (Table 2, columns A and B). It should be noted that immunocapture of epithelial cell (Table 2, column C) increased the detection limit (10 to 100 cells) in comparison with Ficoll separation (1 to 10 cells) (Table 2, column B), indicating a lower assay sensitivity of the first method. mRNA enrichment by nuclei removal and DNase treatment (Table 2, columns D and E) did not further

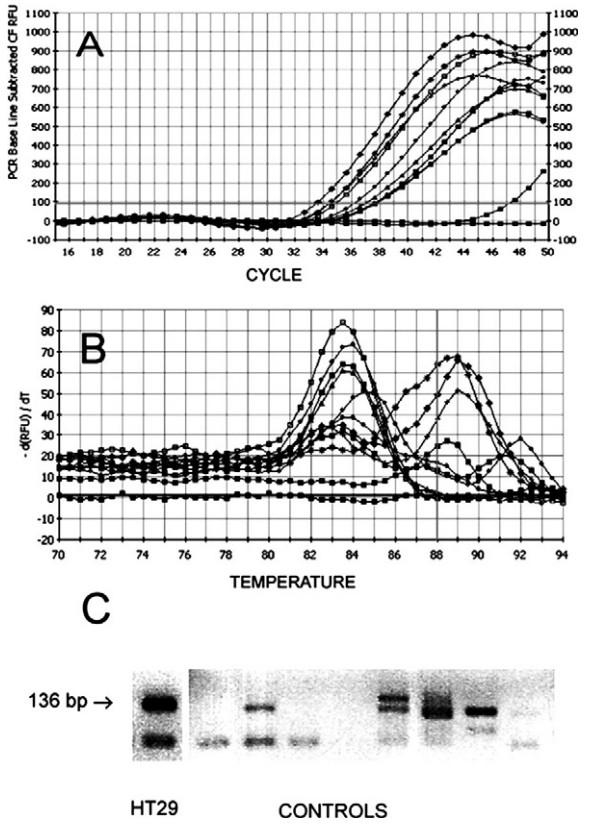

Figure 3. Nonspecific amplifications by conventional intron spanning primers. Amplification plots obtained with cDNA of lympho-monocytes from healthy donors (**A**); melting curves obtained from the same PCR reactions (**B**); gel electrophoresis analysis (2% agarose gel stained with ethidium bromide) of amplified material from lympho-monocytes from healthy donors (controls) and HT29 cells (**C**).

increase the sensitivity of 20N-PCR. As expected, S-PCR was at least 10-fold less sensitive than 20N-PCR (Table 2, column B). Blood containing a low number of spiked HT29 cells was also used to assess the reproducibility of the RNA extraction method. RNA was extracted from blood containing 10 HT29 cells/3 ml in four independent experiments and HT29 cells quantified against a standard curve as described. Calculated interassay CV of the four experiments was 34.2%.

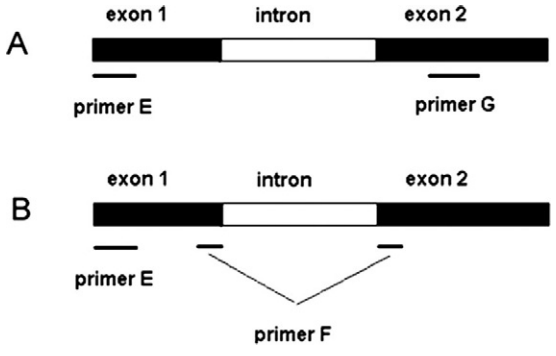

Figure 4. Schematic positions of the intron-spanning primers E and G (**A**) and exon-overlapping primer F (**B**) used for N-PCR.

Table 3. Analytical Variance at Varying Number of HT29 Cells

HT29 cells	C_t*	CV
10,000	13.0 ± 0.29	0.022
1,000	16.2 ± 0.56	0.035
100	19.5 ± 0.37	0.019
10	22.1 ± 0.74	0.034

*Threshold cycles (C_t) are expressed as means ± SD.
20N-PCR assays performed on the same cDNA in six triplicate experiments.

Analysis of Clinical Samples

The 20N-PCR using primers E and F was selected to analyze clinical samples because of its high sensitivity and specificity. Ninety-nine colorectal cancer patients, 110 breast cancer patients, and 150 healthy controls were assayed for circulating cells expressing *CK20*. Tumor staging was performed in all colorectal and breast cancer patients (Table 4). All analyzed controls showed completely negative amplification plots. Conversely, *CK20*$^+$ samples were found in colorectal and breast cancer patients (Table 5). In duplicate experiments, all results were confirmed. Metastatic patients showed higher percentages of positive samples and higher numbers of circulating *CK20* cell equivalents compared with nonmetastatic patients. It should be noted that the number of *CK20* cell equivalents was quantified using a HT29 cells standard curve; differential *CK20* expression could, therefore, interfere with the cell quantification. The association between presence of *CK20* cell equivalents and metastasis was statistically significant for breast cancer patients ($P = 0.026$) but not for colorectal cancer patients ($P = 0.361$). Graphical inspection of the distributions of *CK20* cell equivalents in blood samples of metastatic and nonmetastatic patients showed that data were not normally distributed, with a large number of zero counts in both cancer groups and a few large *CK20* cell equivalents counts in metastatic patients. *CK20* cell equivalents appeared higher in metastatic patients for both cancer types, although statistical significance was achieved only for breast cancer ($P = 0.032$) and not for colorectal cancer ($P = 0.339$). In all *CK20*$^+$ samples, PCR amplification products displayed specific melting profiles, confirming the reliability of the method (Figure 2C). To confirm the above results, all samples were also assayed by a potentially more sensitive, although nonquantitative, analytical method (35N-PCR). All positive samples were confirmed, and no additional positive sample was detected by 35N-PCR, suggesting that 20N-PCR was already maximally sensitive. Negative controls containing all components except cDNA were run in parallel with each series of RT-PCR and did not show any detectable signals, indicating the absence of contamination during these studies.

Discussion

Despite great diagnostic and prognostic interest, the specific presence of metastatic cells in blood of cancer patients is still under debate. *CK20* is considered a marker of neoplastic epithelial cells, but circulating *CK20*$^+$ cells were described to be present or absent in the blood of both cancer patients and healthy subjects ([7,32–35,38]). These apparently conflicting results were not surprising because we (G. Giribaldi, unpublished data) and others[39] frequently obtained false-positive results by using conventional nested RT-PCR methods. Here, the detection limit was improved by a first-round amplification of cDNA by 20 PCR cycles before real-time PCR analysis. First-round PCR amplification conditions were chosen to allow accurate real-time PCR quantification as indicated by the increase in PCR efficiency from 88.9% in S-PCR to 99.0% in 20N-PCR. Under those conditions, the C_t shift of approximately five cycles indicates a theoreti-

Table 4. Union Internationale Contre le Cancer Stage in *CK20*$^+$ and *CK20*$^-$ Colorectal and Breast Cancer Patients

	I	II		IIIA	IIIB	IV	Total
Colorectal cancer							
CK20$^+$	8 (36.4%)	0		0	1 (4.5%)	13 (59%)	22 (100%)
CK20$^-$	18 (23.4%)	3 (3.9%)		12 (15.6%)	7 (9%)	37 (48%)	77 (100%)
Breast cancer		IIA	IIB				
CK20$^+$	6 (18.7%)	10 (31.2%)	5 (15.6%)	3 (9.4%)	0	8 (25%)	32 (100%)
CK20$^-$	40 (51.2%)	14 (18%)	11 (14.1%)	5 (6.4%)	1 (1.3%)	7 (9%)	78 (100%)

Table 5. Case and Control Sample Analysis

	Controls (150)		Breast cancer (110)				Colorectal cancer (99)			
			M		N-M		M		N-M	
			15		95		50		49	
CK20$^+$ patients	yes	no	yes	no	yes	no	yes	no	yes	No
	0	150	8	7	24	71	13	37	9	40
Mean no. of *CK20* cell equivalents	0		5.5		0.9		29.2		4.4	
Median no. of *CK20* cell equivalents	0		0.1		0		0		0	

Distribution of subjects by health status (control, breast cancer, colorectal cancer), presence (M) or absence (N-M) of metastases, and presence/absence of *CK20* cell-equivalents (yes/no). Mean and median number of *CK20* cell equivalents detected in 3 ml of blood.

cal 32-fold increase in sensitivity in comparison with S-PCR. An additional 10-fold decrease of the detection limit was obtained using an intercalating fluorescent dye instead of an oligonucleotide fluorescent probe. The substantial increase in sensitivity made it mandatory to increase specificity to avoid the interference by any nonspecific amplification. Specificity was greatly enhanced using a primer overlapping two adjacent exons that could not anneal to any genomic DNA sequence because its complementary target could be generated only after mRNA splicing. Such novel primer design completely abrogated nonspecific amplifications both in positive samples and negative controls. The present method eliminated the DNase treatment of extracted RNA and allowed the immediate reverse transcription of mRNA into the more stable cDNA. Accuracy was not influenced by dilution of HT29 cells in a large excess of blood, although the detection limit of conventional PCR assays was reportedly influenced by the dilution of target mRNA in blood.[6,31,40] Detection limit was not improved by immunocapture of epithelial cells[41–43] or by increasing first-round cycles,[44,45] suggesting that the sensitivity of the method to detect few RNA copies was already maximal. It should be noted that real-time RT-PCR was performed in a closed system that allowed easier control of DNA contamination compared with conventional PCR. In fact, blanks were always negative in all experiments, indicating lack of cross-contamination by amplified DNA. Despite improved sensitivity, all control samples tested negative for *CK20*$^+$ cells. This result appears to indicate that the *CK20* positivity reported in healthy subjects by others[7,32–35] could more plausibly be of artifactual origin. Twenty-two percent and 29% of all colorectal and breast cancer patients, respectively, had *CK20* cell equivalents in their blood. In breast cancer patients, the association between metastasis and *CK20* cell equivalents in blood was statistically significant. Our results appear to be in accordance with results from others showing a correlation between the number of circulating cancer cells and breast cancer progression.[46] A similar association, albeit not statistically significant, was observed in colorectal cancer patients. Results were highly reproducible and constantly confirmed in duplicate experiments. The range of variation of *CK20* cell equivalent counts was different in the two case series. *CK20* cell equivalents were found per 3 ml of blood as follows: 0 to 40 in nonmetastatic and 0 to 45 in metastatic breast cancer patients, 0 to 147 in nonmetastatic and 0 to 650 in metastatic colorectal cancer patients. We have no explanation for these differences. Possibly, loss of cytokeratin expression may justify apparent loss of sensitivity in less differentiated tumors[47] in the most advanced cases. Varying *CK20* expression in different cancer cells may lead to wrong numbers of *CK20* cell equivalents, which is relative to their *CK20* mRNA content.

In conclusion, we optimized a real-time RT-PCR method, increasing its sensitivity and specificity in comparison with previous PCR methods. No *CK20* cell equivalents were present in the blood of healthy donors, whereas a variable number of *CK20* cell equivalents was detected in the blood of breast and colorectal cancer patients. Because of the absence of false positives, appearance of *CK20* cell equivalents in blood is a strong indication of colorectal or breast cancer. The appearance of *CK20* cell equivalents in blood of patients affected by other tumors remains to be investigated. The prognostic relevance of present data are currently under investigation by a long-term follow-up study of all two-case series patients. We hope that the long-term follow-up of the patients affected by initial tumor stages will give information on the predictive value of circulating *CK20* cell equivalents in the disease progression. Finally, it should be noted that the present method characterized by improved specificity and low detection limits could be easily adapted for detection of minimal residual disease in leukemia and lymphoma as well as for detection of any type of circulating tumor cells where reliable mRNA markers are available.

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