Increased Detection of Circulating Tumor Cells in the Blood of Colorectal Carcinoma Patients Using Two Reverse Transcription-PCR Assays and Multiple Blood Samples


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INTRODUCTION

The objectives of this study were to assess whether the use of two reverse transcription-PCR (RT-PCR) cDNA assays and multiple blood sampling increased circulating tumor cell detection in colorectal cancer patients. Systemic blood was sampled three times at 1-min intervals in 100 colorectal cancer patients (50 primary tumors only and 50 liver metastases), and in 70 control patients without known cancer. After removal of the erythrocytes, samples were subjected to separate RT-PCR reactions using specific primers for carcinoembryonic antigen (CEA) and cytokeratin 20 (CK20). Statistical analysis was performed by the two-sample binomial test and the one-sided McNemar test. There were significant increases in circulating tumor cell positivity when CEA and CK20 assays were used together as compared with either CEA or CK20 assay used alone. There were also significant increases in circulating tumor cell positivity for either CEA or CK20 assay used alone when the results from two blood samples were compared with the results from one sample. Circulating colorectal cancer cell positivity rose from 48% (CEA) and 34% (CK20) with one assay of one sample to 74% when both assays of three samples were used to identify circulating tumor cells. Three non-cancer control patients (4.3%) were positive for either CEA or CK20 assay used alone. There were also significant increases in circulating tumor cell positivity for either CEA or CK20 assay used alone when the results from two blood samples were compared with the results from one sample. Circulating colorectal cancer cell positivity rose from 48% (CEA) and 34% (CK20) with one assay of one sample to 74% when both assays of three samples were used to identify circulating tumor cells. Three non-cancer control patients (4.3%) were positive for either CEA (two patients) or CK20 (one patient). Tumor cells were identified more frequently in the circulation of colorectal cancer patients than had been suggested previously. RT-PCR-based studies of the clinical significance of circulating cancer cells in colorectal cancer should involve multiple blood samples with identification of multiple tumor-related cDNA products.

ABSTRACT

The objectives of this study were to assess whether the use of two reverse transcription-PCR (RT-PCR) cDNA assays and multiple blood sampling increased circulating tumor cell detection in colorectal cancer patients. Systemic blood was sampled three times at 1-min intervals in 100 colorectal cancer patients (50 primary tumors only and 50 liver metastases), and in 70 control patients without known cancer. After removal of the erythrocytes, samples were subjected to separate RT-PCR reactions using specific primers for carcinoembryonic antigen (CEA) and cytokeratin 20 (CK20). Statistical analysis was performed by the two-sample binomial test and the one-sided McNemar test. There were significant increases in circulating tumor cell positivity when CEA and CK20 assays were used together as compared with either CEA or CK20 assay used alone. There were also significant increases in circulating tumor cell positivity for either CEA or CK20 assay used alone when the results from two blood samples were compared with the results from one sample. Circulating colorectal cancer cell positivity rose from 48% (CEA) and 34% (CK20) with one assay of one sample to 74% when both assays of three samples were used to identify circulating tumor cells. Three non-cancer control patients (4.3%) were positive for either CEA (two patients) or CK20 (one patient). Tumor cells were identified more frequently in the circulation of colorectal cancer patients than had been suggested previously. RT-PCR-based studies of the clinical significance of circulating cancer cells in colorectal cancer should involve multiple blood samples with identification of multiple tumor-related cDNA products.

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3 The abbreviations used are: RT-PCR, reverse transcription-PCR; CEA, carcinoembryonic antigen; CK20, cytokeratin 20; DEPC, diethyl pyrocarbonate.
PATIENTS AND METHODS

Patients. Colorectal cancer patients undergoing treatment in one hospital between November 1996 and July 1998 were studied. The diagnosis of primary colorectal carcinoma was confirmed in all cases by endoscopic biopsy, and the primary tumor stage was confirmed by histological examination of the resected primary tumor. All colorectal liver metastasis patients had histological evidence of primary colorectal carcinoma, computed tomography scan evidence of liver metastases, and a subsequent history of liver metastasis growth on serial computed tomography scans associated with a progressive rise in serum CEA level.

Healthy patients with no history of cancer undergoing inguinal hernia repair were recruited as non-cancer controls.

Blood Sampling. To reduce the false positive risk of venesection needle-cored epithelial cells entering the venesectomy needle lumen (7), an i.v. cannula was inserted, and 5 ml of blood were aspirated before sample collection. Three 14-ml samples (designated as samples A, B, and C, respectively) of systemic venous blood were then collected at 1-min intervals from each cancer patient into 7-ml vacutainers (two 7-ml aliquots/14-ml sample) containing sodium EDTA.

A single blood sample was taken via a cannula (as described above) in control patients before inguinal hernia repair.

RNA Extraction from Blood. Blood samples were processed within 15 min of venesection to prevent RNA degradation. The two 7-ml vacutainer aliquots comprising one sample were each added to 35 ml of Erythrocyte Lysis Buffer (Qiagen, Crawley, United Kingdom) and kept on ice for 15 min to allow lysis of RBCs. The nucleated cells, including tumor cells of the resulting solution from each aliquot, were harvested by centrifugation at 1400 rpm (200 × g) for 10 min at 4°C, the supernatant was discarded, 0.5 ml of RNazol B (Biogenesis, Bournemouth, United Kingdom) was added to the residual cell pellets, and the two aliquots from the same sample were then combined. Total RNA was extracted by a modified method of Chomczynski and Sacchi (8) and washed in DEPC-75% ethanol until further analysis. The RNA yield was maximized by the addition of 10 µl of polyniosinic acid solution (16 g/liter) to each sample. All glassware was rinsed in DEPC-water and autoclaved, and solutions were made up in DEPC-water.

RT-PCR. Two µg of total RNA were used for reverse transcription to cDNA. cDNA synthesis was prepared using 100 units of Moloney murine leukemia virus reverse transcriptase (Life Technologies, Inc.), 8 µl of reaction buffer [250 mM Tris-HCl (pH 8.3), 375 mM KCl, and 15 mM MgCl2], and 10 mM each deoxynucleotide triphosphate (Pharmacia, St Albans, United Kingdom). Twenty pmol of random hexamers (Clontech Laboratories, Cambridge, United Kingdom) were added, and primer annealing was performed by incubation for 2 min at 70°C, followed by rapid quenching on ice for 5 min. Reverse transcription was performed by incubating the samples for 1 h at 42°C and then heating them to 94°C for 5 min to inactivate the reverse transcriptase. The final volume of the cDNA reaction sample was 40 µl, and this was increased to 80 µl by adding DEPC-water.

Twenty µl of this cDNA solution were then used for the final PCR reaction after making up to 50 µl by adding PCR buffer (100 mM Tris-HCl and 500 mM KCl; Perkin-Elmer, Warrington, United Kingdom), 2 mM MgCl2 (Perkin-Elmer), 250 µM deoxynucleotide triphosphate (Pharmacia), 2.5 units of AmpliTaq Gold DNA Polymerase (Perkin-Elmer), and 0.5 µM of both sense and antisense primers.

Primer sequences for RT-PCR amplification of CEA (2) were as follows: (a) sense primer, CCATGGGAGTCTCGGCTCGG; and (b) antisense primer, GTAGCTTGCTATGGGCTTGCTTGAT. These CEA primer sequences in the presence of cDNA CEA amplify a 641-bp fragment that does not include other related gene members (Fig. 1; Ref. 9). The conditions for CEA cDNA PCR amplification were 94°C for 10 min to activate the AmpliTaq Gold. This single cycle was followed by 29 cycles at 94°C for 30 s, 56°C for 30 s, and 72°C for 2 min. The final elongation step was 72°C for 10 min.

Primer sequences for RT-PCR amplification of CK20 were as follows: (a) sense primer, CAGACACACGGTGAACGATAGACG; and (b) antisense primer, GATCAGCTTCCACTGTGTTCT. These CK20 primer sequences in the presence of CEA cDNA amplify a 370-bp fragment that does not include other related gene members (Fig. 1). The conditions for CK20 cDNA PCR amplification were 95°C for 10 min to activate the AmpliTaq Gold. This single cycle was followed by 40 cycles at 94°C for 1 min, 60°C for 1 min, and 72°C for 1 min. The final elongation step was 72°C for 7 min (3).

The risk of genomic DNA yielding a product was prevented by designing primers that bridged intronic sequences. RT-PCR primers were synthesized by Oswell (Southampton, United Kingdom).

Identification of RT-PCR Products. PCR products were run on a 1.5% agarose gel electrophoresis and stained with ethidium bromide. Samples that had not been reverse-transcribed did not give rise to product. The identity of the two products had been confirmed previously by Southern blotting (2) and direct sequencing (3).
Each RT-PCR run included a positive control produced from either the HT29 or HT115 colorectal cancer cell line and a negative control in which target cDNA was substituted by nuclease-free water. The quality of the cDNA was assessed using primers for the ubiquitous housekeeping gene β-microglobulin. The RT-PCR method was standardized by loading equal volumes of PCR product on agarose gels to ensure that the intensity of the bands reflected equivalent amounts of detectable product. To assess RNA integrity and quality, formaldehyde RNA gels were run at differing intervals on randomly selected samples. The operator was unaware of the source of each sample while performing the RT-PCR.

Statistical Analysis. Comparisons of the prevalence of tumor cell positivity between groups were performed using the two-sample binomial test (10). A one-sided McNemar test (10) was used to test for equal sensitivity in detection of circulating cancer cells between two sets of results. If different assessment combinations in two sets of results are equivalent, then the same number of positive/negative assessment combination results as negative/positive assessment combination results will occur in each patient. The McNemar test assesses whether one of these outcomes has occurred significantly more frequently than the other. The test was also used to check that results between the three different samples assayed using the same RT-PCR cDNA in the same patient were not significantly different. To avoid inclusion of the same data in both arms of comparisons between single blood and multiple blood samples, statistical differences between the results from samples A and B compared with sample C were assessed. Similarly, where the prevalence of combined CEA and CK20 positivity was compared with that of either one alone, the prevalence of the combination in sample A was compared with that of either one alone in sample C.

The protocol was approved by the Chelsea and Westminster Hospital Research Ethics Committee, and written informed consent was obtained from all patients in the study.

RESULTS

Patients. A total of 100 colorectal cancer patients (male: female, 57:43; mean age, 65.3 years; SD, 9.6 years) were studied. There were 50 patients with primary colorectal carcinomas (Dukes’ stage A or B, 30 patients; Dukes’ stage C, 20 patients) in whom liver metastases were not detected, and there were 50 patients with colorectal liver metastases.

Prevalence of CEA and CK20 cDNA Positivity within a Single Blood Sample. Blood sample A was RT-PCR positive for CEA in 48 patients (25 patients with no liver metastases) and was RT-PCR positive for CK20 cDNA in 34 patients (16 patients with no liver metastases; Table 1). There were no significant differences (McNemar tests) in the patterns of positivity for either CEA or CK20 in sample A compared with either sample B or C, or in sample B compared with sample C (Table 2).

Effect of Combined CEA and CK20 Detection on the Prevalence of RT-PCR Positivity. The number of patients in whom blood sample A was positive for either CEA or CK20 (56 patients) was significantly greater (binomial, \( P = 0.007 \)) than that when CEA alone was examined in sample C (39 patients) (Fig. 2). There were significantly more (McNemar test, \( P = 0.002 \)) patients who were either CEA or CK20 positive in sample A who were not detected by CEA alone in sample C compared with CEA alone in sample A not detected by CEA or CK20 in sample C.

Similarly, the number of patients in whom blood sample A was positive for either CEA or CK20 was significantly greater (binomial, \( P = 0.0004 \)) than that positive when CK20 only was examined in sample C (33 patients). There were significantly more (McNemar test, \( P = 0.011 \)) positive patients for either CEA or CK20 in sample A who were not detected by CK20 alone in sample C compared with CK20 alone in sample A not detected by CEA or CK20 in sample C.

Effect of Multiple Blood Samples on the Prevalence of RT-PCR Positivity. If a positive result was defined either as when samples A or B or sample C was positive, then the number of CEA-positive patients rose significantly (binomial, \( P = 0.007 \)) from 39 when sample C was positive to 56 when samples A or B were positive. There were significantly more (McNemar test, \( P = 0.004 \)) patients positive for CEA in samples A or B who were not detected by CEA in sample C, compared with the number detected by CEA in sample C but not in samples A and B.

Similarly, the number of CK20-positive patients rose significantly (\( P = 0.004 \)) from 33 for sample C to 51 when sample A or B was positive. There were also significantly more (\( P = 0.003 \)) CK20-positive patients in samples A or B who were not detected by CK20 in sample C, compared with those detected by CK20 in sample C but not in samples A and B.

Combined Effect of Multiple Blood Samples and Both CEA and CK20 Estimation. The number of patients in whom blood sample A or B was positive for CEA or CK20 (67 patients) was significantly greater (binomial, \( P = <0.0001 \)) than the number of patients positive when CEA only was examined in sample C (39 patients). There were significantly more (McNemar test, \( P < 0.0001 \)) patients positive for CEA or CK20 in samples A or B who were not detected by CEA alone in sample C, compared with the number detected by CEA alone in sample C but not by CEA or CK20 in samples A and B.

Similarly, the number of patients in whom blood sample A or B was positive for CEA or CK20 was also significantly greater (\( P < 0.0001 \)) than that positive when CK20 only was examined in sample C (33 patients). There were significantly more (\( P < 0.0001 \)) patients positive for CEA or CK20 in samples A or B who were not detected by CK20 alone in sample C, compared with those detected by CK20 alone in sample C but not by CEA or CK20 in samples A and B.

Significant differences were obtained for equivalent comparisons between other sample combinations (sample A with

<table>
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<tr>
<th>Table 1</th>
<th>Circulating cell positivity by cDNA (CEA or CK20) used in RT-PCR identification of circulating cancer cells in each of three blood samples examined</th>
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<tbody>
<tr>
<td>RT-PCR</td>
<td>cDNA</td>
</tr>
<tr>
<td>---------</td>
<td>------</td>
</tr>
<tr>
<td>CEA</td>
<td>100</td>
</tr>
<tr>
<td>CK20</td>
<td>100</td>
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0.002) patients who were either CEA or CK20 positive in sample A who were not detected by CEA alone in sample C compared with CEA alone in sample A not detected by CEA or CK20 in sample C.

Each RT-PCR run included a positive control produced from either the HT29 or HT115 colorectal cancer cell line and a negative control in which target cDNA was substituted by nuclease-free water. The quality of the cDNA was assessed using primers for the ubiquitous housekeeping gene β-microglobulin. The RT-PCR method was standardized by loading equal volumes of PCR product on agarose gels to ensure that the intensity of the bands reflected equivalent amounts of detectable product. To assess RNA integrity and quality, formaldehyde RNA gels were run at differing intervals on randomly selected samples. The operator was unaware of the source of each sample while performing the RT-PCR.
samples B and C and sample B with samples A and C) for both CEA and CK20.

**Combined Effect of Multiple Blood Samples and Both CEA and CK20 Estimation on Circulating Cancer Cell Prevalence by the Presence of Primary Colorectal Cancer or Liver Metastasis.** Significantly more patients with primary tumor only (McNemar test, \( P < 0.002 \)) but not patients with liver metastases (\( P = 0.191 \)) were positive in samples A or B for CEA or CK20 compared with negative in sample C to CEA alone than were positive in sample C to CEA alone and negative in samples A or B for CEA or CK20 (Fig. 3). Significantly more patients with primary tumor only (McNemar, \( P = 0.048 \)) and patients with liver metastases (\( P = 0.021 \)) were positive in samples A or B for CEA or CK20 compared with negative in sample C for CK20 alone than were positive in sample C for CK20 alone and negative in samples A or B for CEA or CK20.

When a positive was defined as any positive result for CEA or CK20 occurring in any of the three samples examined, the number of positive patients rose to 74 (36 patients with primary tumor only and 38 patients with liver metastasis).

**Non-Cancer Control Patients.** A positive result in the single blood sample from 70 healthy individuals before hernia repair occurred in three patients (CEA, 2 patients; CK20, 1 patient).

**DISCUSSION**

Depending on the cDNA assessed, there was a 17–23% increase in positivity for circulating cancer cells when two blood samples compared with one blood sample were examined (Fig. 2). We have previously shown that false positive results can arise from needle coring of epithelial cells, expressing epithelial antigens such as CEA or CK20 cDNA, into the blood (7), and it has also been reported that cells expressing cDNA for CK20 can be identified in the blood of non-cancer patients with colitis (11). Although false positive results were identified in our healthy non-cancer control patients, these were not of sufficient magnitude (2.9% per blood sample for CEA and 1.4% per blood sample for CK20) to account for the increased positivity observed with multiple blood sampling in the colorectal cancer patients. The findings were more consistent with circulating cancer cells being either absent or below the detection threshold in some blood samples from colorectal cancer patients in whom other samples were positive. Experimental animal model studies

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Table 2  Positivity by cDNA (CEA or CK20) used in RT-PCR identification of circulating cancer cells in each of three blood samples (A, B, or C) from 100 colorectal cancer patients

<table>
<thead>
<tr>
<th>Sample</th>
<th>CK20-positive patients</th>
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<tbody>
<tr>
<td></td>
<td>A only</td>
</tr>
<tr>
<td>CEA-positive (patients)</td>
<td></td>
</tr>
<tr>
<td>A only</td>
<td>3</td>
</tr>
<tr>
<td>B only</td>
<td>0</td>
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<tr>
<td>C only</td>
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<td>A and B</td>
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<td>B and C</td>
<td>0</td>
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<tr>
<td>A, B, and C</td>
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</tr>
<tr>
<td>None</td>
<td>1</td>
</tr>
<tr>
<td>Total (CK20)</td>
<td>9</td>
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**Fig. 2** Cumulative increase in the number of patients whose blood was found to be RT-PCR positive by the number of blood samples/patient examined (1, sample C; 2, samples A and B; 3, samples A–C) for CEA and CK20 cDNA (each cDNA individually or cDNA positive).
have suggested that circulating cancer cells are aggregated in clumps of varying size (6), and our findings supported this in colorectal cancer patients by demonstrating that sufficient tumor cells for a positive test were not invariably present in all blood samples from patients in whom circulating cells were identified within some blood samples. It is also possible that despite precautions, variations in detection sensitivity occurred between samples (12).

The positive rate for a single mRNA marker examined in a single sample was similar in the present study to previous reports (2–5) in colorectal cancer. However, heterogeneity of sample positivity by type of mRNA marker did occur. Where a single blood sample was examined for both CEA and CK20 cDNA, the prevalence of positivity to both was only 26%, but when three samples were examined, the prevalence doubled to 53% of patients being positive for both markers. One explanation for this increase could be a variation in the amounts of CEA and CK20 mRNA obtained from tumor cells within each sample, resulting in a negative result for one marker within a single sample sometimes being associated with a positive result for the other marker within the same sample. As a result of these sample-to-sample mRNA variations, there was a significant increase in the detection of circulating cancer cells where assays for two mRNA markers rather than one mRNA marker were used. We have previously reported (2) an absence of correlation between the prevalence of CEA mRNA positivity within a single sample/patient and serum CEA levels.

Identifying a colorectal cancer patient with circulating cancer cells by a positive result from any of three blood samples for either of the two mRNA markers resulted in a doubling of the positive rate (Fig. 2), suggesting that tests based on a single sample for one mRNA marker only (2, 3, 4, 5) may underestimate the prevalence of circulating cancer cells in colorectal cancer. This positivity increase was observed both in patients with primary tumor only and, to a lesser extent, in the case of patients with CEA in liver metastasis (Fig. 3).

Despite this, circulating cancer cells were not detected in 22% of liver metastasis patients. Possible explanations for this failure to detect circulating cancer cells in patients with disseminated colorectal cancer are that circulating cancer cells were present but were below the detection threshold in these patients or were absent from the circulation at the times of blood sampling. Studies involving repeated blood sampling over a longer time period in these patients would help to resolve this. The identification of circulating tumor cells in 72% of primary colorectal cancer patients in whom metastases had not been identified suggests that circulating tumor cells may occur in colorectal cancer patients who are cured by primary tumor removal. This is consistent with studies involving animal models of small implanted tumor foci (13) that suggest that cancer cells can be detected within the circulation early after tumor implantation and before the development of micrometastases. Additional studies of the clinical significance of circulating cancer cells in colorectal cancer involving multiple blood samples and identification of more than one tumor-related mRNA marker are needed to evaluate this further.

Although the accuracy of the present methods has been estimated by in vitro studies (2, 3) in which blood samples from healthy individuals without cancer were “spiked” with colorectal cancer cells, our results must be interpreted with caution in the absence of a reliable validating test for the identification of circulating tumor cells in cancer patients. Comparisons of RT-PCR-based methods with other sensitive techniques being developed (14) for identification of circulating cancer cells would address this. However, the present results suggest that tumor cells are more frequently present in the circulation of colorectal cancer patients than reported previously (2–5).

REFERENCES


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