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DETECTION OF COLORECTAL CARCINOMA CELLS IN CIRCULATING PERIPHERAL BLOOD BY REVERSE TRANSCRIPTION-POLYMERASE CHAIN REACTION TARGETING CYTOKERATIN-20 mRNA

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Summary

For the detection of circulating colorectal carcinoma cells, we investigated the presence of cytokeratin 20 (CK 20) mRNA in the peripheral blood of colorectal carcinoma patients. Application of our published technique resulted in analysis by reverse transcription followed by three-step nested polymerase chain reaction. This analysis could detect a single Colo 205 colon cancer cell mixed with 1 ml of blood. Our system also successfully detected the presence of CK 20 mRNA in actual patients' peripheral blood samples. Our highly sensitive and specific system for the detection of CK-20 mRNA from patients' peripheral blood thus seems to be useful for screening for circulating colorectal carcinoma cells.

Key Words: cytokeratin-20 mRNA, RT-PCR, colorectal carcinoma, hematogenous dissemination

Colorectal carcinoma is one of the most frequently occurring cancers and its high incidence of hematogenous metastasis and/or recurrence poses a clinically serious problem. To screen preoperative patients with a high risk of recurrence and to identify postoperative patients with impending recurrence, we

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have focussed on the presence of cytokeratin 20 (CK 20). CK-20 polypeptide is an "intermediate-sized filament", an important constituent of the mammalian cytoskeleton and its differential expression is closely linked with specific programs of differentiation of different cell types (1). It has already been established that the expression of CK 20 is almost entirely confined to the gastric and intestinal epithelium, urothelium, and Merkel cells, and not to normal hematopoietic cells (2). CK-20 expression in carcinomas is reported to resemble that seen in corresponding normal epithelia of origin (2). In the case of colorectal adenocarcinoma, CK 20 is known to be expressed in the vast majority of carcinomas, including all grades of differentiation and malignancy, and also in metastatic tumors (2,3). Detection of CK-20 mRNA by reverse transcription-polymerase chain reaction (RT-PCR) from the peripheral blood of colorectal carcinoma patients is therefore likely to be useful for screening for the presence of colorectal carcinoma cells in circulation. Burchill et al have shown that this is possible by using a high CK-20 expressing colon cancer cell line mixed into healthy donor's blood, but their system required at least 100 cells per 1 ml of blood (4). Studies to capture circulating carcinoma cells by using specific target sequences have already been performed on various carcinomas (5-9). We have also developed a highly sensitive RT-PCR system to identify hematogenously disseminating hepatocellular carcinoma cells in concentrations as low as one cell per 1 ml of blood (10,11) and also for gastric and pancreatic carcinoma cells (12). For the clinical application of this "epithelial-specific" CK 20 to the detection of carcinoma cells in circulation, the present study represents a major improvement in the sensitivity of the system resulting from the use of nested RT-PCR. The effectiveness of the system has been confirmed with blood samples of colorectal carcinoma patients.

Patients and Methods

Patients All patients' profiles are listed in Table I. Diagnosis of recurrence was made by echography, computed tomography, chest radiography and, if necessary, angiography.

RNA extraction and cDNA synthesis RNA was extracted from peripheral blood according to the acid guanidinium-phenol-chloroform (AGPC) method (13) with a slight modification. In short, 5ml of heparinized whole blood was mixed well with 5ml of a guanidinium isothiocyanate-enriched solution D [6M-guanidinium isothiocyanate/ 25mM-sodium citrate (pH7.0) / 0.5% Sarcosyl / 100mM β -mercapto-ethanol]. We increased the quantity of guanidinium isothio-cyanate to the level higher than the one previously described (10) for better protection of RNA in the whole blood according to

TABLE I

Sex (M/F)	Age at test	Clinical findings	Resected organs	Timing of blood sampling	CK 20 mRNA	Out-come
1. M	54	CK[#1] with LM[#2]	Colon and Liver	pre[#3]	+	after 11Mo[#4] R[#5] (Lung)
2. F	58	RK[#6] with LM, Lung and Renal M[#7]	None	pre	+	n.d.[#8]
3. F	39	CK, M(-)	Colon	pre	+	after 11Mo R(+)
4. F	61	LM of CK (9M from Colectomy)	Liver	pre	+	after 11Mo R(Liver)
5. F	72	CK with Peritoneal dissemination	None	pre	+	n.d.
6. M	49	RK, M(-)	Rectum	pre	+	after 1Mo R(-)
7. M	53	RK, M(-)	Rectum	pre	-	after 15Mo R(-)
8. M	58	CK, M(-)	Colon	pre	-	after 15Mo R(-)

[#1] CK :colon cancer
[#2] LM :liver metastasis
[#3] pre :before operation
[#4] Mo :months
[#5] R :recurrence
[#6] RK :rectal cancer
[#7] M :metastasis
[#8] n.d. :not determined

the report of Gillespie et al (14). Two ml of this sample mixture, which corresponds to 1ml of total blood, was further processed by the ordinary AGPC method. Extracted RNA was solubilized in diethyl pyrrocarbonate (DEPC)-treated water (14) and was reverse-transcribed in a 50μ l mixture consisted of 10 μ l of 5× buffer (Gibco BRL), 2mM dNTP (Wako Pure Chemical Industries,LTD,Japan),10mM DTT (Gibco), 0.25μ g random hexamer (Pharmacia), 5μ g bovine serum albumin (BSA)(Gibco) and 200U M-MLV reverse transcriptase (Gibco, Cat.No.28025-013). The reverse transcription was performed at 37 ℃ for one hour.

<u>Preparation of positive control template</u> A human colon cancer cell line, Colo 205 (purchased from American Type Cell Culture) (15) was cultivated in Dulbecco's modified eagle's medium supplemented with 10% fetal bovine serum. mRNA was extracted from Colo 205 cells using a Quick Prep® mRNA purification kit

(Pharmacia) and then reverse transcription was performed using 1 μg of mRNA according to the above method.

PCR primers The specific primers for CK-20 gene detection were synthesized according to the published sequence (4). Sense primer 1 (5'-CAGACACACGGTGAACTATGG-3') within exon 1 and antisense primer 2 (5'-GATCAGCTTCCACTGTTAGACG-3') within exon 3 are both identical with the published sequences by Burchill, et al (4). For the nested PCR, we used our original sense primer 3 (5'-CTGTTTGTTGGCAATGAGAAAATGG-3') within exon 1 and antisense primer 4 (5'-GTATTCCTCTCTCAGTCTCATACT-3') spreading over exon 2 (the 3' two bases, C and T) and exon 3.

PCR protocol First step-PCR was performed using primers 1 and 2 which amplify a 370-bp fragment (4). The 100μl of individual PCR mixture was composed of one half of the reverse-transcribed sample (25μl) and 10 μl of 10 × PCR buffer (Perkin-Elmer-Cetus, Norwalk,CT), 200pmole of each primer in 4 μl of TE(pH8.0), 2 μg of BSA, 0.4mM dNTP and DEPC-treated water. The reaction mixture was overlaid with mineral oil, heat-denatured at 93 ℃ for 8 minutes and then cooled to 80 ℃ for addition of 5 units of Taq polymerase (AmpliTaq®, Perkin-Elmer-Cetus). The first-step PCR consisting of 30-second denature (94℃), followed by 1-minute annealing (63 ℃) and then by 30-second chain extension (72 ℃) was repeated 35 cycles. The second set of primers consisted of primers 1 and 4 which amplify a 349-bp fragment. The 50 μl of the second-step PCR mixture was composed of one 5 μl of the first PCR product, 4.5μl of 10 × PCR buffer, 100 pmole of each primer, 1 μg of BSA, 0.2 mM dNTP and DEPC-treated water. The reaction mixture was overlaid with mineral oil, heat-denatured at 93 ℃ for 8 minutes and then cooled to 80℃ for addition of 2.5 units of Taq polymerase (AmpliTaq ®, Perkin-Elmer-Cetus). The second-step PCR consisting of 1-second denature (94 ℃), followed by 20-second annealing (63 ℃) and then by 10-second chain extension (72 ℃) was performed for 35 cycles. 2μl of this second PCR product was used for the third PCR. The third set of primers consisted of primers 3 and 4 which amplify a 303-bp fragment. The 50 μl constituent of the third PCR mixture was basically the same as the second PCR mixture except for the difference in the template and the primers. The amplification program for the third PCR was also basically the same as the first PCR except for the annealing temperature set to 62℃. 10μl of the PCR product was subjected to electrophoresis on 2.5% agarose gels (Agarose NA,Pharmacia Biotech,Sweden) containing 20ng/ml ethidium bromide.

To test the reliability of RNA extraction, a 319-bp β-actin cDNA fragment was amplified using the 50-pmole-each primer pair reported by Fuqua et al (14). The PCR template was the residual

one half of sample cDNA. The constituent of the PCR mixture was the same as the mixture for CK 20 mRNA except for the difference of primers. Except for the annealing temperature (55℃) calculated from the primer sequences, our PCR protocol for the detection of β-actin cDNA fragment was the same as that for the detection of CK-20 cDNA fragment. When the 35-cycle amplified band was faint, one-fiftieth of the PCR product (corresponding to 1μl) was amplified in a new PCR mixture for 30 cycles using the same primer set under the same program. We were able to observe the amplification of β-actin mRNA in all the samples from the patients and samples from the controls without fail (data not shown).

The positive control for CK-20 mRNA detection was the PCR performed with 0.1μg of Colo 205 mRNA. The negative control for the first step was the simultaneously-performed PCR without any template. The negative control for the second- and third-step was the nested PCR product performed in the same way with the sample PCR using previous negative control sample as the template.

Results

PCR amplification of Colo 205-derived cDNA As can be seen in Fig.1, the first, second and third PCR products were visible as a individual, clear, single bands. Non-specific additional bands were not observed on either the positive lanes or the negative control lanes.

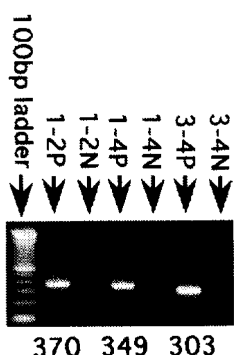

FIG.1

PCR amplification of Colo 205 cDNA forms a clear single band. P indicates PCR product with template and N without template. Numbers 1,2,3 and 4 represent the name of the primer. A 100-bp ladder was used as a marker, the lowest being a 100-bp band.

Detection of CK-20 mRNA in serially diluted Colo 205 cDNA To determine the sensitivity of our assay, 0.1 μg of Colo 205 cDNA was serially diluted and used as the PCR template. The

first PCR visualized a 10^{-2}-diluted sample (Fig.2A), and the second PCR a 10^{-5}-diluted sample (Fig.2B). However the third PCR did not increase the sensitivity. Considering the outstanding ability of the second and third primer pairs to amplify targeted sequences in the positive control sample (Fig.1), we thought this lower-than-expected sensitivity might be the result of the low production of CK-20 mRNA by Colo 205 cells. To confirm this hypothesis, we serially diluted the

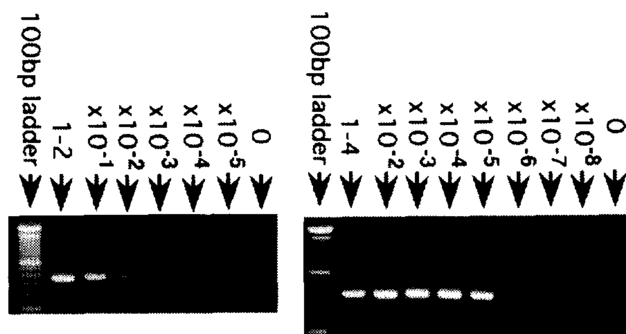

FIG.2A FIG.2B

PCR using serially-diluted Colo-205 derived cDNA to test the sensitivity. Fig.2A represents the first PCR product and Fig.2B the second. 1-2 and 1-4 represent the positive controls for the respective primer pairs.

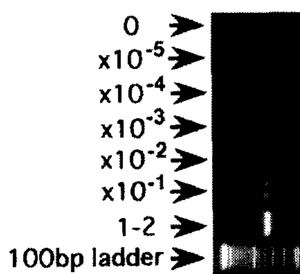

FIG.3

Electrophoresis of the serially-diluted first-step PCR product, using 0.1μ g of Colo 205-derived cDNA as the template. 1-2 represents the positive control.

first PCR product by using 0.1μ g of Colo 205 mRNA as the template. As is seen in Fig.3, these serially-diluted first PCR products produced bands with optical densities similar to those of the bands produced by samples which were serially diluted and then amplified. We then performed the second PCR by using individual serially diluted first PCR products as the template. Then, using this second one as the template, we performed the third PCR. The second PCR visualized a sample diluted up tp 10^{-7} (Fig.4), and the third one up to 10^{-8} (Fig.5) which seemed to

be the limit of the existence of the template. Thus, as long as the template remained, our system showed a powerful sensitivity.

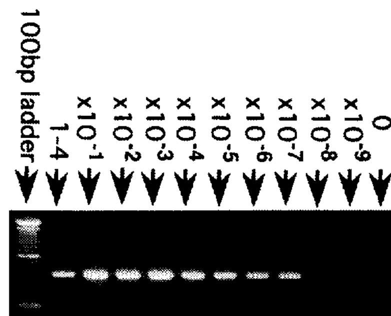

FIG.4
The second-step PCR amplification of the serially-diluted first-step PCR product. 1-4 represent the positive control.

Detection of CK-20 mRNA in a small quantity of Colo 205 cells mixed with 1ml of normal healthy volunteer's blood Volumes of 10^4, 10^3, 10^2, 10, 5, 2 or 1 Colo 205 cells were mixed with 1ml of healthy volunteer's blood. Total RNA extraction, cDNA synthesis and PCR amplification were performed in an identical manner as for patients' blood. The second PCR visualized a 10^4-cell sample (data not shown) and the third PCR a sample with only one cell (Fig.6).

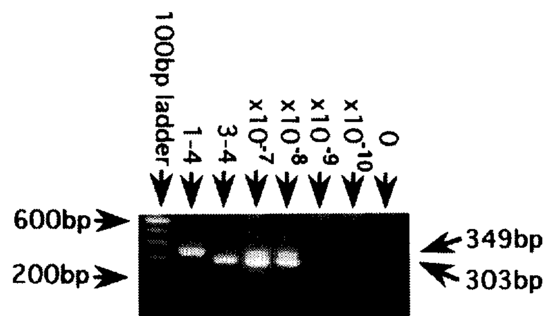

FIG.5
The third-step PCR amplification of the serially-diluted first-step PCR product. 1-4 and 3-4 represent the positive controls for the pairs of primers.

Detection of CK-20 mRNA in patients' samples Of the eight patients with colorectal carcinoma, six were positive for CK-20 mRNA in their peripheral blood (Table I and Fig.7). All these patients were found to be positive only after the third PCR. All four patients with distant metastases or recurrence (No.1, 2, 4 and 5) were positive. Patient No.2 had simultaneous metastases to the liver, lung and kidney, and patient No.5 peritoneal dissemination. Patient No.1 showed simultaneous

liver metastasis and patient No.4 liver metastasis which had become apparent 9 months after colectomy. In spite of hepatectomy, these two CK-20 mRNA-positive patients developed a second metastasis after 11 months either in the lung or in the liver.

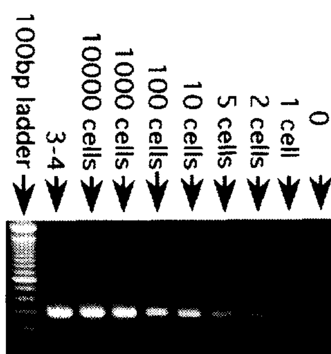

FIG.6
Even one Colo 205 cell mixed in 1 ml of healthy volunteer's blood was detected at the third PCR. 3-4 represents the positive control.

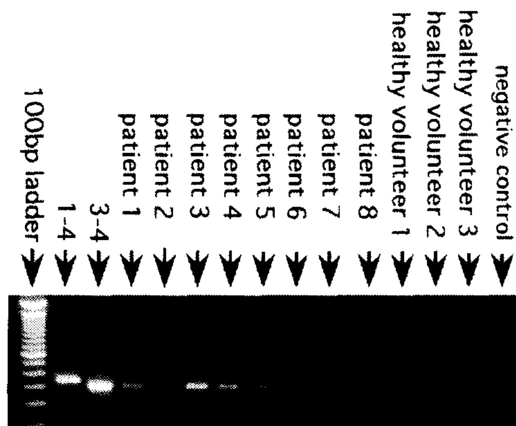

FIG.7
The third-step PCR amplification of colorectal carcinoma patients' samples (1-8) and healthy volunteers' samples (normal 1-3). 1-4 and 3-4 represent the positive controls for respectively the second and third PCR.

Among the preoperative patients without distant metastasis, two patients were positive for CK-20 mRNA (No.3 and 6). Patient No.3 was found to have recurrence in the peritoneal cavity after 11 months, and patient No.6 received the primary operation just one month ago. Two patients negative for CK-20 mRNA (No.7 and 8) remained negative for recurrence during the 16-month observation period.

None of the three healthy volunteers was positive (Fig.7).

Sequencing of the final PCR product of the positive control sample and the randomly-chosen patients' samples revealed that the amplified products were consistently identical with the expected cDNA sequence (data not shown).

Discussion

We have developed a highly sensitive RT-PCR system to detect CK-20 mRNA in the peripheral blood. CK 20 is known to produce a specific expression in epithelial cells (4) and has also been identified as being expressed in almost all colorectal carcinoma cells (1,2). Therefore, the presence of CK-20 mRNA in the peripheral blood of colorectal carcinoma patients, that is, the presence of CK-20-expressing cells in circulation, can reasonably be expected to suggest the existence of hematogenously disseminating colorectal carcinoma cell(s).

Different from the previous one-step PCR system (4) which required as many as 100 cells to detect the presence of a high CK-20 expressing colon cancer cell line, our nested PCR system using additional original primers was able to detect a single CK-20 expressing cell mixed with 1 ml of CK-20 negative, healthy donor's blood.

In addition to this high sensitivity, our system succeeded in achieving high specificity for the actual colorectal patients' blood. Patients with metastasis or recurrence were consistently positive for CK-20 in their peripheral blood. Of four preoperative patients without metastasis, two were CK-20 mRNA negative and they remained recurrence-free for the 16-month observation period. The remaining two patients were positive for CK-20 mRNA. One of these patients received the primary operation only one month ago, and the other developed recurrence 11 months after the analysis. Taking these results into consideration, we consider this recently operated patient may be at high risk for recurrence and that careful follow-up and/or adjuvant therapy may be necessary.

Although the possibility of contamination of a few normal epithelial cells, as previously mentioned, cannot be ruled out because of the lack of a definite marker to discriminate between cancer cells and non-cancerous cells, non-cancerous epithelial cells cannot possibly live long without anchoring, and CK-20 mRNA was not found in healthy donors' blood in either our study or those of other investigators (1,2).

In conclusion, our nested RT-PCR detection of CK-20 mRNA from

peripheral whole blood seems to provide practical and useful information on the hematogenous dissemination of colorectal carcinoma cells.

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