Potential Usefulness of Detecting Cyclooxygenase 2 Messenger RNA in Feces for Colorectal Cancer Screening

SHIGERU KANAOKA,* KEN–ICHI YOSHIDA,* NAOYUKI MIURA,† HARUHIKO SUGIMURA,§ and MASAYOSHI KAJIMURA*

*First Department of Medicine, †Department of Biochemistry, and §Department of Pathology, Hamamatsu University School of Medicine, Hamamatsu, Japan

Background & Aims: **Cyclooxygenase 2 (COX-2) is overexpressed frequently in aerodigestive tumors, especially in colorectal tumors. Therefore, it may be a suitable biomarker for colorectal cancer (CRC) screening. We performed a pilot study of whether detecting COX-2 expression in fecal RNA enables us to discriminate between patients with and without CRC.** ***Methods:*** **The study cohort included 29 patients with CRC, and 22 control patients without neoplastic disease of the colon or rectum. RNA was isolated from routinely collected stool samples using a modified method. The expression levels of carcinoembryonic antigen (CEA) and COX-2 were determined by nested reverse-transcription polymerase chain reaction (RT-PCR).** ***Results:*** **The sensitivity and the specificity of fecal COX-2 assay for CRC were 90% (95% confidence interval [CI], 73%–98%) and 100% (95% CI, 85%–100%), respectively, whereas those of the fecal CEA assay for CRC were 100% (95% CI, 88%–100%) and 5% (95% CI, 2%–23%), respectively. COX-2 messenger RNA (mRNA) was detected in 3 of 4 patients with Dukes' stage A, 13 of 14 patients with Dukes' stage B, and 10 of 11 patients with Dukes' stage C or D. COX-2 mRNA was detected in 5 of 7 patients with proximal cancer and in 21 of 22 patients with distal cancer. The COX-2 assay was superior to the CEA assay for detecting CRC in terms of specificity, although both assays had high sensitivity.** ***Conclusions:*** **This fecal COX-2 assay had high sensitivity and high specificity for detecting CRC. These results suggest that it would be a promising approach for detecting CRC, although a larger study is necessary to assess the sensitivity and the specificity.**

Colorectal cancer (CRC) is the second most common cause of death in the Western world, and is the most common fatal cancer among nonsmokers.[1,2] In the United States, CRC accounts for 11% of all cancers, with an estimated 130,200 new cases and 48,100 deaths in the year 2001,[1] and in Japan there are 85,000 annual cancer registrations and 35,600 deaths caused by this disease.[3] Because a large number of patients can be treated successfully if CRC is detected early, it is important to make an early diagnosis. Colonoscopy and sigmoidoscopy are highly specific and sensitive tests for neoplasia, but they are invasive and limited by patient compliance and physician availability.[5] The fecal occult blood test is a noninvasive and simple examination that has been shown to reduce the incidence, morbidity, and mortality of CRC.[6–9]

Recently, methods for the isolation of human DNA directly from stool samples have allowed the analysis of genetic alterations associated with neoplasia.[10–15] Because these genetic alterations are associated directly with the development of neoplasia, they have clear advantages over indirect markers such as fecal occult blood. The disadvantage of DNA-based stool assays is the lack of sensitivity caused by clonal heterogeneity in CRC.[16,17]

RNA-based stool assays have been reported in several preliminary studies,[18–21] one of which showed that CD44 variant expression in human feces could be detected in 68% of CRC patients by a combination of reverse-transcription polymerase chain reaction (RT-PCR) and Southern hybridization.[21] These results prompted us to develop a more sensitive assay. The major disadvantage of this RNA-based stool assay is that it is difficult to isolate RNA without degradation and it is difficult to remove impurities from feces such as PCR inhibitors. A protocol that isolates RNA with less degradation and that can detect CRC molecular markers therefore is required. The ideal target molecules for an RNA-based stool assay are those that are expressed only in CRCs and not in normal mucosa. Cyclooxygenase 2 (COX-2) gene expression is increased frequently in aerodigestive tumors, including esophagus, stomach, pancreas, lung, and colon.[22] It has been shown that COX-2 is overexpressed in more than 80% of CRCs compared

Abbreviations used in this paper: CEA, carcinoembryonic antigen; CI, confidence interval; COX-2, cyclooxygenase 2; CRC, colorectal cancer; RT-PCR, reverse-transcription polymerase chain reaction.

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Table 1. Characteristics of CRC and Control Patients

	CRC patients	Control patients
n	29	22
Sex (M/F)	20/9	11/11
Age (*yr*)[a]	71 (50–85)	66.5 (20–85)
Total RNA (*μg/g of stool*)[a]	70 (5–414)	55.5 (4–379)

[a]Median (range).

with normal colonic mucosa.[23–25] Therefore, COX-2 is considered a good candidate gene for an RNA-based stool assay.

The purpose of our study was to develop a test based on using fecal RNA to detect CRC. We describe here the modified methods used to isolate RNA quickly and with sufficient purity to assay by RT-PCR, allowing us to distinguish CRC patients from control subjects by COX-2 messenger RNA (mRNA) expression levels.

Materials and Methods

Patients and Samples

All CRC patients and controls in this study were admitted to Hamamatsu University School of Medicine between 1999 and 2002. We evaluated 29 patients with primary colorectal adenocarcinoma who were diagnosed colonoscopically and histologically. The median age of the patients with CRC was 71 years (range, 50–85 yr), and 20 of the 29 patients were men (Table 1). Twenty-five of 29 patients underwent surgical resection of their primary tumor, 2 patients underwent endoscopic resection, and 2 patients had tumors that were considered inoperable because of metastasis to other organs. The tumors were classified according to Dukes' staging, yielding stage A (n = 4), stage B (n = 14), stage C (n = 8), and stage D (n = 3) (Table 2).

A total of 22 control patients who had no neoplastic lesions colonoscopically also were included in this study (11 men, 11 women; median age, 66.5 yr; range, 20–85 yr) (Table 1). The reasons for performing colonoscopy in the control patients included lower abdominal pain, anemia, constipation, or CRC screening, showing that the control patients in this study were not an average-risk population.

Stool samples were collected before colonoscopy from all of the control patients and 3 of the 29 patients with CRC, and before surgery from the remaining 26 patients with neoplasia, all of whom initially were diagnosed colonoscopically in the outpatient unit. Their samples were collected more than 2 weeks after colonoscopy. The samples were stored at 4°C immediately after collecting and transferred to a freezer set at −80°C within 6 hours. The samples were stored for up to 2 years before isolating RNA. This study was approved by the institutional local genetic research ethics committee at Hamamatsu University School of Medicine. Oral and written

RNA Isolation From Feces

To develop an RNA-based stool assay for the detection of CRC, we needed a new method to isolate fecal RNA of sufficient quantity, quality, and purity. The procedure we developed for isolating RNA from feces uses a combination of Isogene (Nippon Gene, Toyama, Japan) and RNeasy kit (Qiagen GmbH, Hilden, Germany). Approximately 1 g of frozen fecal pellet was added to a sterile 5-mL tube containing 3 mL Isogene, and homogenized with a Handy Microhomogenizer (Microtech Nition, Chiba, Japan) for a few minutes. After homogenization, the slurry was poured into sterile 1.5-mL tubes, which were centrifuged at 12,000*g* for 5 minutes at 4°C. The supernatant from each tube was transferred carefully to new sterile 1.5-mL tubes. To each tube, 0.3 mL Isogene and 0.3 mL chloroform were added, the tubes were shaken vigorously for 30 seconds, incubated for 5 minutes at 4°C, and centrifuged at 12,000*g* for 15 minutes at 4°C.

The aqueous phase from each tube was removed carefully without contamination from the interface and transferred to a fresh 1.5-mL tube. An equal volume of 70% ethanol was added, and the tubes were vortexed vigorously for 30 seconds. The mixed solution (700 μL) was added to an RNeasy minispin column (Qiagen GmbH), and the columns were

Table 2. Characteristics of CRC Patients

Patient no.	Age, *yr*	Sex	RNA, *μg*	Location	Dukes' stage	Size, *cm*	CEA assay[a]	COX-2 assay[a]
1	64	F	5	A	C	6.5	(+)	(+)
2	77	F	34	A	B	7.0	(++)	(+)
3	78	M	70	R	D	5.0	(++)	(+++)
4	65	M	35	R	A	6.5	(+)	(+)
5	69	M	82	C	C	5.0	(++)	(−)
6	55	F	63	S	B	5.2	(+)	(+)
7	83	M	89	C	B	2.7	(++)	(+)
8	55	F	62	R	B	2.5	(++)	(+)
9	70	M	13	R	B	5.0	(+)	(+)
10	73	M	63	R	A	1.5	(+)	(++)
11	64	F	9	S	D	3.5	(+)	(+++)
12	85	M	8	D	C	3.3	(+)	(+)
13	71	M	105	R	C	7.3	(++)	(+)
14	75	M	155	R	C	5.5	(++)	(+)
15	50	M	12	R	B	4.2	(+)	(+)
16	74	M	110	S	B	4.8	(+)	(+)
17	55	M	31	R	A	1.2	(+)	(−)
18	82	M	9	S	C	3.2	(+)	(+)
19	82	M	13	S	B	4.0	(+)	(+)
20	63	M	179	S	C	3.2	(+++)	(+++)
21	52	F	117	A	C	4.3	(+)	(+)
22	72	M	252	R	B	7.0	(+)	(++)
23	60	M	67	R	B	3.6	(++)	(+)
24	80	F	195	S	A	2.2	(++)	(++)
25	75	M	237	C	B	4.2	(++)	(+++)
26	69	M	414	R	B	5.0	(+)	(++)
27	68	M	117	R	D	4.3	(+++)	(+++)
28	76	F	77	S	B	6.5	(+++)	(+++)
29	83	F	358	A	B	4.5	(++)	(−)

[a]RT-PCR was graded as negative (−); weakly positive (+); positive (++); or strongly positive (+++).

centrifuged at 10,000*g* for 15 seconds at room temperature. The remaining steps were performed according to the manufacturer's instructions. Total RNA concentrations were determined by ultraviolet spectrophotometry, and the RNA samples were stored at −80°C.

RT–PCR

Complementary DNA (cDNA) was synthesized using ReverScript II (Wako Chemical, Osaka, Japan) with 1 μg fecal RNA and 250 ng random hexamers according to the manufacturer's instructions, and amplified using nested PCR. The cycling conditions were as follows: carcinoembryonic antigen (CEA), 95°C for 5 minutes, followed by 20 cycles at 95°C for 1 minute and 72°C for 2 minutes; COX-2, 95°C for 5 minutes, followed by 20 cycles at 95°C for 1 minute, 56°C for 1 minute, and 72°C for 1 minute. The nested PCR reactions were as follows: 95°C for 5 minutes, followed by 25 cycles at 95°C for 1 minute, 69°C was used for CEA, and 56°C was used for COX-2 for 1 minute, and 72°C for 1 minute. CEA primers were as described previously[26] and COX-2 primers were designed according to published sequence information.[27] The primers used were as follows: CEA A primer: 5′–TCTGGAACTTCTCCTGGTCTCTCAGCTGG-3′; CEA B primer: 5′–TGTAGCTGTTGCAAATGCTTTAAGGAAGAAGC-3′; CEA C primer: 5′–GGGCCACTGCTGGCATCATGATTG-3′; COX-2 A primer: 5′–CTGAAACCCACTCCAAACACAG-3′; COX-2 B primer: 5′–ATAGGAGAGGTTAGAGAAGGCT-3′; COX-2 C primer: 5′–GCACTACATACTTACCCACTTCAA-3′.

Primers A and B were used for the first round of PCR, and primers C and B were used for the second round. To distinguish from contaminated genomic DNA, we selected both forward and reverse primers at different exons. The 131-bp CEA PCR product and the 178-bp COX-2 PCR product were identified by electrophoresis of 10 μL through 4% NuSieve 3:1 agarose (BioWhittaker Molecular Applications, Rockland, ME) in Tris-acetate-ethylenediaminetetraacetic acid buffer and ethidium bromide staining. Negative controls for the RT-PCR consisted of either a reverse-transcribed sample without total RNA or PCR mixture only. To ensure reproducibility of results, all samples were reverse transcribed and amplified in triplicate. In addition, the fidelity of both the 131-bp CEA PCR product and the 178-bp COX-2 PCR product from the stool samples was confirmed by DNA sequencing.

Statistical Analysis

Sensitivity and specificity were estimated relative to the results of colonoscopy in the usual manner; 95% confidence intervals (CIs) for these estimated parameters were based on the exact binominal distribution. Statistical significance was determined by the Fisher exact test, the Mann–Whitney test, the Kruskal–Wallis test, and Spearman coefficient by rank test. *P* values less than 0.05 were interpreted as statistically

Table 3. Comparison of COX-2 Assay With CEA Assay

	COX-2 assay	95% CI	CEA assay	95% CI
Sensitivity	90% (26/29)	73%–98%	100% (29/29)	88%–100%
Specificity	100% (22/22)	85%–100%	5% (1/22)	2%–23%

Results

RNA Isolation

We modified the method of Chomczynski and Sacchi[28] by using both Isogene and RNeasy kit. After purification, the RNA was analyzed to detect CEA and COX-2 expression. The median yield of RNA was 70 μg (range, 5–414 μg) per gram of stool from cancer patients and 55.5 μg (range, 4–379 μg) per gram of stool from control patients, respectively (Tables 1 and 2). There was no significant difference in RNA yield between the 2 groups ($P > 0.05$, by the Mann–Whitney test), and the RNA isolation took only 1 hour using our method.

Nested RT-PCR to Detect CEA

CEA mRNA was detected in stool samples from all cancer patients (100%; 95% CI, 88%–100%) and in all but one control patient (95%; 95% CI, 77%–98%) (Tables 2 and 3). Representative results are shown in Figure 1. There was no significant difference on CEA expression levels in feces between CRC patients and control patients ($P > 0.05$, by the Mann–Whitney test). Further, the factors such as Dukes' stage, location, or size of the tumor had no influence on CEA expression levels in feces ($P > 0.05$, by the Kruskal–Wallis test). This revealed that CEA is not suitable for use in an RNA-based stool assay to detect CRC, however, detecting CEA mRNA could prove the isolation of RNA was sufficient to assay RT-PCR.

Nested RT-PCR to Detect COX-2

We performed RNA-based stool assays unblinded to the clinical data. Because COX-2 expression has been found in nearly 20% of normal mucosa,[25] we preliminarily needed to prepare various quantities of cDNA made from control patients for the first round of PCRs. We assayed the first round of PCR using 3 different amounts of cDNA, the 75%, the 45%, and the 15% part of cDNA synthesized from 1 μg of fecal RNA. When the 75% quantity of cDNA was used for the first round of PCR, COX-2 mRNA was detected in 2 of 22 control patients (data not shown). However, when assayed using less cDNA, COX-2 mRNA was detected in none of the control patients. So we decided to use the 45% quantity of cDNA synthesized from 1 μg of fecal RNA for the

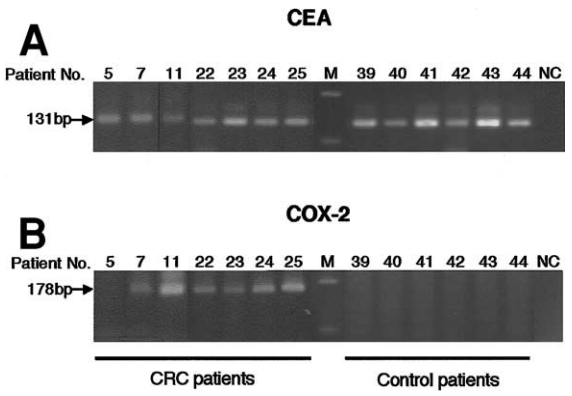

Figure 1. Nested RT-PCR for CEA and COX-2. (*A*) *Lanes 1–7* show CEA nested RT-PCR results from patients with CRC and *lanes 8–13* show those from control patients. All lanes show a PCR product of the expected length of 131 bp. (*B*) The same samples were analyzed for COX-2 mRNA. *Lanes 2–7* show a PCR product of the expected length of 178 bp. *Lane 1* and all lanes from control patients (*lanes 8–13*) show negative results. *Lanes 1–7* corresponds to patient numbers 5, 7, 11, and 22–25 in Table 2, respectively. M, 100-bp ladder size marker; NC, negative control.

the 29 stool samples from patients with CRC (90%; 95% CI, 73%–98%). None of the 22 control samples showed positive results (0%; 95% CI, 0%–15%; $P < 0.0001$, by the Fisher exact test) (Tables 2 and 3). The representative results are shown in Figure 1.

Although CRC patients were predominately men (69%), positive results were obtained similarly in both 18 of 20 male patients and 8 of 9 female patients (Tables 1 and 2). When patients were assigned according to Dukes' classification, positive results were obtained in 3 of the 4 patients with stage A cancer, 13 of the 14 patients with stage B cancer, 7 of the 8 patients with stage C cancer, and all 3 of the patients with stage D cancer. Positive results were obtained in 4 of 5 patients with small-size tumors (1.2–3 cm), 16 of 18 patients with medium-size tumors (3.1–6 cm), and in all 6 patients with large-size tumors (>6 cm). It is noteworthy that 5 of 7 patients with cancer proximal to the splenic flexure had positive results, as did 21 of 22 patients with more distal cancer. There was no correlation between CEA expression levels and COX-2 expression levels in feces from CRC patients ($P > 0.05$, Spearman coefficient by rank test) or between COX-2 expression levels in feces from CRC patients and Dukes' stage, location, or size of the tumor ($P > 0.05$, by the Kruskal-Wallis test). There was no significant difference in COX-2 expression levels in feces between men and women ($P > 0.05$, by the Mann–Whitney test). In conclusion, the COX-2 assay was superior to the CEA assay in terms of specificity (100% vs. 5%), although both assays had high sensitivity (90% vs. 100%)

Discussion

The data presented herein show the potential use of RT-PCR to detect COX-2 mRNA in feces from CRC patients using the appropriate primers and assay conditions. In the past decade, a number of studies have reported neoplasm-specific DNA changes in feces from patients with CRC or large adenomas.[10–15] The DNA-based stool assays used in these studies typically have analyzed mutations on either a single gene or more than 2 genes. Because the gene mutations were specific to neoplasms with an exemption such as K-*ras*, these assays were virtually 100% specific. Indeed, a multitarget DNA-based assay recently has been reported to yield 91% sensitivity and 100% specificity, when analyzed in 22 patients with CRC and 28 subjects with endoscopically normal colons. After recovery of human DNA from stool using a sequence-specific hybrid capture technique, assay components targeted point mutations at any of 14 mutational hot spots on *p53*, and *APC* genes, mutations on BAT-26, microsatellite instability marker, and highly amplifiable DNA.[12]

It is known that neoplasms continuously exfoliate luxuriant populations of viable colonocytes, unlike the sparse and largely apoptotic cells shed from normal mucosa.[29] Whole colonocytes have been recovered from stool samples,[30] and isolated fecal colonocytes can be incorporated into assay systems using immunocytochemical methods or RT-PCR assays.[21,31] However, cytolytic factors in stools may compromise the stability of sloughed colonocytes.[32] To address these factors, we devised a method of isolating RNA from feces by homogenizing a frozen fecal pellet in the presence of guanidine salt, thereby inactivating both cytolytic factors and RNase in stools and removing PCR inhibitors. As a result, RNA could be isolated more efficiently (in a less degraded condition) and more rapidly (approximately 1 h), allowing specific RT-PCR assays for both CEA and COX-2 to be performed. A technique to recover colonocytes from stool with an average yield of more than 10^6 cells per gram of stool and with the viability rate as high as 80% has been reported previously.[30] Theoretically, 10^6 viable cells could produce approximately 10 μg of total RNA. It also was shown that 5–30 μg of RNA per gram of stool from cancer patients and approximately 5 μg of RNA per gram of control stool were obtained.[20] Our yields of fecal RNA from both cancer and control patients were much higher than in this previous report, and there was no significant difference between cancer patients and control patients in terms of yield (70 μg, 55.5 μg/g of stool, respectively). Therefore, most of the RNA

flora, with the proportion of RNA from colonic epithelium being considered only a small part. Consequently, we needed to amplify both CEA and COX-2 mRNA by nested PCR instead of standard PCR methods.

Serum CEA protein currently is used clinically to monitor the management of colorectal carcinoma. The gene for CEA is one of the most widely expressed genes in cancer cells.[33] It is expressed in 95% of colorectal, gastric, and pancreatic cancers, similarly being present in columnar epithelial cells such as normal colon and stomach. Expression of CEA mRNA in normal surface epithelium is almost similar to that of epithelial cells of colorectal carcinoma patients.[34] We hypothesized that expression levels of fecal CEA mRNA of CRC patients would be higher than that of control patients. Contrary to expectation, there was no significant difference in expression levels between the 2 groups, indicating that the mRNA extracted from stool samples using our method was sufficient for RT-PCR but that the CEA molecule is not suitable for RNA-based stool assays to detect CRC.

COX-2 gene expression is up-regulated in most colorectal carcinomas compared with surrounding normal mucosa by approximately 50-fold.[23] Immunohistochemical analysis detected COX-2 in cancer cells, inflammatory mononuclear cells, vascular endothelial cells, and fibroblasts.[25] Furthermore, exfoliation of colonocytes and leukocytes are quantitatively greater from CRC epithelia than from normal mucosa.[29] We selected COX-2 as a molecular marker for detecting CRC by our method, and succeeded in detecting fecal COX-2 mRNA from the CRC patients with high sensitivity. To our surprise, this assay had 90% sensitivity and 100% specificity, however, this extraordinary specificity came from only 22 control patients. We will need to examine a larger number of control patients for estimating specificity of this assay. We found no significant difference between levels of COX-2 mRNA and Dukes' stage, location, or size of the tumors. Therefore, it is conceivable that recovery of fecal colonocytes from CRC patients, even those with proximal colon cancer, would be sufficient to detect fecal COX-2 mRNA from small tumors. We performed a COX-2 assay on 26 cancer patients using stool samples obtained more than 2 weeks after colonoscopy. It is not certain whether colonoscopy together with forceps biopsy can influence the effect of COX-2 expression. Further evaluation is needed to clarify this issue.

In summary, it is possible to detect COX-2 mRNA in feces from patients irrespective of the clinical stage of CRC. Moreover, our method has certain advantages compared with the fecal occult blood test. Only a single fecal pellet is sufficient for the assay and more sensitive in terms of analyzing a single molecule. It currently remains unclear, however, that the fecal COX-2 assay is a useful screening test for CRC in the clinical setting. A larger study remains to be performed. First, to assess the sensitivity for a much broader spectrum of tumors, including early Dukes' A cancer as well as premalignant adenoma; second, to assess the specificity for a much broader spectrum of controls, in which possible cause of the false-positive results should be clarified; and, finally, to determine whether the fecal COX-2 assay is as sensitive and specific as the fecal occult blood test in average-risk persons. When these issues are solved, our approach would be attractive for CRC screening.

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