Fecal Cyclooxygenase 2 Plus Matrix Metalloproteinase 7 mRNA Assays as a Marker for Colorectal Cancer Screening

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Abstract

We previously reported that fecal cyclooxygenase 2 (COX-2) mRNA assay, detecting COX-2 mRNA in feces, is useful for identifying subjects with colorectal cancer (CRC). To further improve the sensitivity, we evaluated the usefulness of the combination of COX-2 mRNA and matrix metalloproteinase 7 (MMP-7) mRNA assays as a marker of CRC. The study cohort included 62 patients with CRC and 29 control patients without colorectal neoplasia. RNA was isolated from routinely collected fecal samples. The expression levels of COX-2 and MMP-7 mRNAs were determined by nested reverse transcription-PCR. PCR conditions were optimized where the specificity of fecal COX-2 and MMP-7 mRNA assay result in 100%. The sensitivity of each fecal assay was 87% [95% confidence interval (95% CI), 76-94%] and 65% (95% CI, 51-76%) for CRC, respectively. The sensitivity of fecal RNA test (either marker being positive) was high for CRC (90%; 95% CI, 80-96%). The sensitivity of the fecal RNA test was also high (93%; 95% CI, 80-98%) in patients with stage I or II who are often cured by surgical resection. The fecal RNA test using COX-2 and MMP-7 mRNAs improved the sensitivity to detect CRC without decreasing the specificity. These results suggest that the fecal RNA test would be a promising approach for CRC screening, although larger clinical investigations are indicated. (Cancer Epidemiol Biomarkers Prev 2009;18(6):1888-93)

Introduction

Colorectal cancer (CRC) is one of the most common causes of death in the Western world. In the United States, CRC accounts for 11% of all cancers, with an estimated 153,760 new cases and 52,180 deaths in the year 2007 (1). In Japan, there are 94,500 annual cancer registrations and 38,900 deaths attributable to this disease (2). Early diagnosis of CRC is crucial because the cure rate for patients without metastases is high. Colonoscopy and sigmoidoscopy are highly specific and sensitive tests for neoplasia of the colon, but they are invasive and are limited by patient compliance and physician availability (3). The fecal occult blood test (FOBT) is a noninvasive and simple examination, and has reduced the incidence, morbidity, and mortality associated with CRC (4-7). However, only less than 10% of people who have positive FOBT actually have CRC (8). The test may also not be suitable for screening of precancerous adenomas, in which bleeding often does not occur (9). Thus, there is a need for newer fecal tests with better performance characteristics for noninvasive screening of CRC.

Recent advances in isolation of DNA directly from stool samples have allowed the analysis of genetic alterations associated with neoplasia (10-16). One study showed that the fecal DNA test is superior to guaiac-based FOBT for detecting CRC (16). Because genetic alterations are associated directly with the development of neoplasia, such tests have clear advantages over indirect markers such as FOBT.

RNA-based stool assays have been reported in several preliminary studies (17-21). We previously reported the usefulness of detecting cyclooxygenase 2 (COX-2) mRNA in feces to identify the patients with CRC and adenoma (20, 21). The overexpression of COX-2 was reported to be found in 80% to 90% of adenocarcinoma and 50% to 60% of adenomas (22, 23). Therefore, it is necessary to add other biomarkers to the assay for improving the sensitivity of the screening for CRC and adenoma.

Matrix metalloproteinases (MMP) are zinc-dependent endopeptidases, which act in the degradation of extracellular matrix during cancer progression. Overexpression of MMP-1, MMP-2, MMP-3, MMP-7, MMP-9, MMP-13, and MMP-14 has been shown in human CRC (24). Expression of MMP-7 mRNA in CRCs and adenomas is 50 and 43 times higher, respectively, than the level in adjacent normal mucosa (25). MMP-7 expression is affected by the degree of dysplasia, with focal expression in low-grade dysplasia and more widespread expression in high-grade dysplasia (26, 27). MMP-7 was localized along the apical/luminal border of tumor cells in APCMin/+ mice (28), suggesting that overexpression of MMP-7 mRNA may be detected by measuring mRNA in feces. In human CRCs, expression and localization of MMP-7 were distinct from those of COX-2 (29), and the degree of the expression of these two mRNAs were not statistically correlated (30). Therefore, we hypothesized that MMP-7 may be a...
Materials and Methods

Patients and Samples. The institutional local genetic research ethics committee at Hamamatsu University School of Medicine (Hamamatsu, Japan) approved this study. Oral and written informed consent was obtained from all patients. The studied population consisted of 62 patients with CRC who were diagnosed colonoscopically and histologically. A total of 29 patients in whom no pathologic findings were observed were served as control. Stool samples were collected at between 2 and 4 wk after diagnostic colonoscopy with a few biopsies. Stool sample collection was done before endoscopic or surgical resection of CRC. Collected samples were stored at 4°C immediately after collection and transferred to a −80°C freezer within 24 h. The samples were stored for up to 2 y before isolating RNA. The median age of cancer patients and control patients was 70 y (range, 48-86 y) and 68 y (range, 20-85 y), respectively. Patients’ profiles are shown in Table 1. CRCs were classified according to the International Union Against Cancer tumor-node-metastasis classification (31).

Immunochromatic FOBT. All stool samples were examined using a single immunochromatic FOBT (IFOBT) with Magstream Hem Sp (Fujirebio, Inc.), an immunochromatic test for human hemoglobin. The IFOB tests were done at the laboratory in the hospital independently from the authors.

RNA Isolation from Feces. RNA was isolated from fecal samples using a previously published method.(20) Briefly, 3 mL Isogen (Nippon Gene) were added to sterile 5-mL tubes each containing ∼0.5 g frozen fecal pellet. Pellets were homogenized for a few minutes using a Handy Microhomogenizer (Microtech Niti-on). The slurries were then poured into sterile 1.5-mL tubes, and centrifuged at 12,000 × g for 5 min at 4°C. The supernatants were transferred carefully to new sterile 1.5-mL tubes. To each tube, 0.3 mL Isogen and 0.3 mL chloroform were added. The tubes were shaken vigorously for 30 s and then centrifuged at 12,000 × g for 15 min at 4°C. The aqueous phase from each tube was removed carefully, avoiding contamination from the interface, and transferred to fresh 1.5-mL tubes. An equal volume of 70% ethanol was added, and the tubes were vortexed vigorously for 30 s. The mixed solution (700 μL) was added to an RNeasy minispin column (Qiagen GmbH), and the columns were centrifuged at 8,000 × g at room temperature. The remaining steps were done according to the manufacturer’s instruction. Total RNA concentrations were determined by UV spectrometry, and the RNA samples were stored at −80°C.

Reverse Transcription-PCR. cDNA was synthesized using Reverse Transcriptase M-MLV (RNase H−; Takara Bio, Inc.) with 1 μg fecal RNA and 250 ng random hexamers according to the manufacturer’s instructions, and amplified using nested PCR. For the first-round PCR for carboxyembryonic antigen (CEA) and COX-2, we used 15% of cDNA synthesized from 1 μg fecal RNA. For the first-round PCR for MMP-7, 45% of cDNA synthesized from 1 μg fecal RNA was used. The cycling conditions for the first-round PCR were as follows: CEA, 95°C for 5 min, followed by 20 cycles at 95°C for 1 min and 72°C for 2 min; COX-2, 95°C for 5 min, followed by 20 cycles at 95°C for 1 min, 56°C for 1 min, and 72°C for 1 min; MMP-7, 95°C for 5 min, followed by 20 cycles at 95°C for 1 min, 55°C for 1 min, and 72°C for 1 min. The nested PCR reactions were as follows: CEA, 95°C for 5 min, followed by 25 cycles at 95°C for 1 min, 69°C for 1 min, and 72°C for 1 min; COX-2, 95°C for 5 min, followed by 20 cycles at 95°C for 1 min, 56°C for 1 min, and 72°C for 1 min; MMP-7, 95°C for 5 min, followed by 35 cycles at 95°C for 1 min, 55°C for 1 min, and 72°C for 1 min. CEA and COX-2 primers were as described previously (20, 32) and MMP-7 primers were designed according to published sequence information. The primers used were as follows:

CEA A primer: 5′-TCTGGAACCTTTCCTCTGGTCTCTT- CAGCTGG-3′;
CEA B primer: 5′-TGAGCTGGTCTGGCAATCTT- TAAGGAAGAACGC-3′;
CEA C primer: 5′-GGGCCCATTGGGCACTGAT- GATG-3′;
COX-2 A primer: 5′-CTGAAACCCTTCCCCATCTC- CAGCTGG-3′;
COX-2 B primer: 5′-ATAGAGAAGGTGATTAGA- GAAGGCT-3′;
COX-2 C primer: 5′-GCACTACATATTACCCATCTC- CAA-3′;
MMP-7 A primer: 5′-ATGAGTGACTACAGTGGGA- AA-3′;
MMP-7 B primer: 5′-AAAATGCAGGGGATCTCTT- TG-3′;
MMP-7 C primer: 5′-TAAAACCTTCCGCTCATT- GA-3′;
MMP-7 D primer: 5′-TCGATCCACTGTAATATGCGC- GG-3′.

Table 1. Characteristics of cancer and control patients

<table>
<thead>
<tr>
<th>Location</th>
<th>Cancer patients</th>
<th>Control patients</th>
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<tbody>
<tr>
<td>Cecum</td>
<td>6</td>
<td>29</td>
</tr>
<tr>
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<td>12</td>
<td>12</td>
</tr>
<tr>
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</tr>
<tr>
<td>Descending colon</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>Sigmoid colon</td>
<td>17</td>
<td>17</td>
</tr>
<tr>
<td>Rectum</td>
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<td>20</td>
</tr>
<tr>
<td>Size (mm)</td>
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<td></td>
</tr>
<tr>
<td>Small (10-29)</td>
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<td>14</td>
</tr>
<tr>
<td>Medium (30-49)</td>
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<td>24</td>
</tr>
<tr>
<td>Large (≥50)</td>
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cent good combination marker for COX-2 in screening CRC, and evaluated the usefulness of the combination of COX-2 and MMP-7 mRNAs called “fecal RNA test” in this study. We also compared the sensitivity and specificity of our fecal RNA test with those of the FOBT for detecting CRC patients.
used for the first round of PCR, and primers C and D were used for the second round. We selected both forward and reverse primers to different exons. PCR products for CEA, COX-2, and MMP-7 (131, 178, and 166 bp, respectively) were identified by electrophoresis of 10 μL through 4% agarose gel, stained with ethidium bromide, and visualized with UV transillumination using the FAS-II system (Toyobo). Negative controls for the reverse transcription-PCR reactions 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 duplicate. In this study, a positive fecal RNA test result was defined as being positive for the fecal COX-2 mRNA assay or for the fecal MMP-7 mRNA assay.

### Statistical Analysis

In the present study, sensitivity is defined as the ratio of a positive test among cancer patients and specificity is defined as the ratio of a negative test among control patients. Sensitivity and specificity were estimated relative to the colonoscopy results; 95% confidence intervals (95% CI) for these estimated parameters were based on the F distribution. Statistical significance between the result of fecal COX-2 mRNA assay and that of fecal MMP-7 mRNA assay and between the results of the fecal RNA test and those of IFOBT were determined by the exact binomial test. The Spearman rank correlation coefficient (r) between the degrees of COX-2 and MMP-7 mRNAs expression was calculated using the statistical software SPSS version 12.0. P values <0.05 were taken as being statistically significant. All statistical tests were two-sided.

### Results

#### Immunochemical FOBT

IFOBTs were positive in 45 of the 62 CRC patients (73%; 95% CI, 60-83%). The IFOBT sensitivity in our samples was 38% (95% CI, 14-68%), 81% (95% CI, 62-94%), 91% (95% CI, 59-100%), and 73% (95% CI, 39-94%) for stage I, II, III, and IV tumors, respectively. Three control patients were false positive for IFOBT; thus, the specificity of IFOBT was 90% (95% CI, 73-98%; Table 2A).

#### Optimization of PCR

To get 100% specificity of fecal COX-2 mRNA assay and fecal MMP-7 mRNA assay, we needed to prepare various quantities of cDNA made from control patients for the first round of PCR and cycle numbers of nested round PCR. We assayed the nested round of PCR for COX-2 using two kinds of cycle numbers, the 25 cycles and the 20 cycles. When the 15% quantity of cDNA synthesized from 1 μg fecal RNA for the first round of PCR and the 25 cycles for the nested round of PCR was used, COX-2 mRNA was detected in 2 of 29 control patients (data not shown). However, when assayed using 20 cycles, COX-2 mRNA was detected in none of the control patients. Thus, we decided to use the 20 cycles.
for the nested round of PCR for COX-2. We assayed the first round of PCR for MMP-7 using three kinds of quantity of cDNA, the 75%, the 45%, and the 15% part of cDNA synthesized from 1 μg fecal RNA. When the 75% quantity of cDNA was used for the first round of PCR, a diffuse smear was produced upon agarose gel electrophoresis (data not shown). However, when assayed using less amount of cDNA, neither target fragment of MMP-7 nor a diffuse smear upon agarose gel electrophoresis was detected in control patients. Thus, we decided to use the 45% quantity of cDNA synthesized from 1 μg fecal RNA for the first round of PCR for MMP-7.

Detection of CEA mRNA in Feces from Cancer Patients and Control Patients. CEA mRNA was detected in stool samples from all cancer patients (100%; 95% CI, 95-100%) and from all but one control patient (97%; 95% CI, 82-100%; Fig. 1). There was no significant difference in CEA expression levels in feces between CRC patients and control patients. As described previously (20), CEA is not a suitable biomarker for detecting CRC; however, detecting CEA mRNA could verify that sufficient RNA was isolated to assay by reverse transcription-PCR.

Detection of COX-2 mRNA and MMP-7 mRNA in Feces from Patients with CRC. COX-2 mRNA was detected in 54 of the 62 stool samples from CRC patients (87%; 95% CI, 76-94%; Table 2A; Fig. 1). The fecal COX-2 mRNA assay sensitivity for stage I, II, III, and IV tumors was 77% (95% CI, 46-95%), 96% (95% CI, 81-100%), 82% (95% CI, 48-98%), and 82% (95% CI, 48-98%), respectively.

MMP-7 mRNA was detected in 40 of the 62 stool samples from CRC patients (65%; 95% CI, 51-76%; Table 2A; Fig. 1). The fecal MMP-7 mRNA assay sensitivity for stage I, II, III, and IV tumors was 38% (95% CI, 14-68%), 76% (95% CI, 58-91%), 73% (95% CI, 39-94%), and 55% (95% CI, 23-83%), respectively.

Combination of Fecal COX-2 mRNA Assay and Fecal MMP-7 mRNA Assay (Fecal RNA Test). The sensitivity of the fecal COX-2 mRNA assay for CRC was significantly higher than that of the fecal MMP-7 mRNA assay (87% versus 65%, P = 0.001). However, the fecal MMP-7 mRNA assay detected two cancer patients who were negative by the fecal COX-2 mRNA assay. When both fecal COX-2 mRNA assay and fecal MMP-7 mRNA assay were combined, the sensitivity of fecal RNA test was 90% (95% CI, 80-96%) for CRC (Table 2A). The sensitivity in patients with stage I or II was significantly higher than that of IFOBT (93% versus 68%, P = 0.006), and the sensitivity in patients with stage I was 77% (95% CI, 46-95%).

The tumors were classified into three categories based on size: small (10-29 mm in diameter, n = 14), medium (30-49 mm in diameter, n = 24), and large (at least 50 mm in diameter, n = 24); fecal RNA test was most sensitive for detecting tumors in the large category. However, the sensitivity of fecal RNA test was relatively high for detecting tumors in the small category (86%; 95% CI, 57-98%; Table 2B).

There was an overlap between each fecal test–positive cases and IFOBT–positive cases (Table 2C). IFOBT was positive in three of six stool samples from CRC patients whose fecal RNA test using COX-2 and MMP-7 mRNAs was negative; however, fecal RNA test using COX-2 and MMP-7 mRNAs was positive in 14 of 17 stool samples from CRC patients whose IFOBT was negative.

Correlation between Fecal COX-2 mRNA Assay with Fecal MMP-7 mRNA Assay. We graded the level of expression of fecal COX-2 mRNA and MMP-7 mRNA as follows: negative (−), weakly positive (+), positive (++), and strongly positive (+++). The correlation coefficient (r) between fecal COX-2 mRNA and MMP-7 mRNA was 0.58 (P < 0.01).

Discussion

In this study, we showed that the sensitivity of the fecal RNA test using COX-2 and MMP-7 mRNAs is high for CRC (90%; 95% CI, 80-96%) and that the fecal RNA test is positive in 93% of subjects with stage I or II. These findings suggested that fecal RNA test is useful to detect the subjects with CRC, including the early-stage tumors that are often curable by surgical resection.

IFOBT is widely accepted as a noninvasive and simple method for detecting CRC, and thereby is used widely. The introduction of FOBT reduced cumulative CRC mortality by 15% to 33% during the subsequent 10 to 14 years (4-6). In a recent review, the sensitivity of FOBT using guaiac-based tests for CRC detection was reported as 26% in asymptomatic nonreferred populations (33). IFOBTs were recommended for CRC screening due to the higher sensitivity than guaiac-based tests (7, 34-36).

In fact, a single IFOBT was reported to have 65.8% sensitivity for detecting in invasive CRC (37). Because of this high
sensitivity, we used a single IFOBT for comparison with our new fecal RNA test. The 73% sensitivity achieved in our study using a single IFOBT was slightly higher than that reported previously (35, 37), probably reflecting that our subjects tested in this study included more advanced CRC with ulceration. Generally, the efforts to increase the sensitivity of FOBT result in the decrease in its specificity (38). Because there is no fecal blood level cutoff that yields satisfactory specificity and sensitivity for colorectal neoplasia, new screening methods is required to develop.

In the last decade, a number of studies have reported neoplasms-specific DNA changes, up-regulation of some genes, and epigenetic changes in feces from patients with CRC or advanced adenomas (10-21, 39). A multitarget fecal DNA-based assay was recently reported to yield ~90% positive results. In that study, following recovery of human DNA from stool using a sequence-specific hybrid capture technique, assay components targeted point mutations at any of 15 mutational hotspots on K-ras, TP53, and APC genes; mutations on BAT-26, microsatellite instability marker; and highly amplifiable DNA (12).

In another study, fecal DNA test targeting 21 mutations had 51.6% sensitivity in a prospective manner, whereas the sensitivity of guaiac-based FOBT was only 12.9% (16). The fecal vimentin methylation assay was reported to have 46% sensitivity (43 of 94 cancer patients) and 90% specificity (178 of 198 cancer-free individuals; ref. 39).

In the present study, the sensitivity of fecal COX-2 mRNA assay for detecting CRC was higher than that of a single IFOBT. Although the fecal MMP-7 mRNA assay was not sensitive for CRC detection than a single IFOBT, the addition of MMP-7 mRNA assay to the fecal COX-2 mRNA assay increased the sensitivity for CRC. The sensitivity of fecal RNA test using COX-2 and MMP-7 mRNAs was significantly higher than that of a single IFOBT for detecting patients with stage I or II that are likely to be cured by surgical resection. Furthermore, the sensitivity of fecal RNA test using COX-2 and MMP-7 mRNAs for detecting patients with tumors of small size was superior to IFOBT. These findings suggested that this fecal RNA test is better than IFOBT in identifying subjects with small colorectal tumors.

In general, a low correlation coefficient (r) between two markers suggests a good combination for increasing the sensitivity of the test. At first, we evaluated several markers such as MMP-7, MMP-2, Ets-related transcription factor (ELAF), and c-myc that are reported to be up-regulated in early to mid colorectal carcinogenesis as candidates for combination markers with COX-2 (25, 30, 40, 41); however, none of these markers except MMP-7 had high sensitivity and high specificity for CRC. In this study, the correlation coefficient (r) between COX-2 and MMP-7 expression in feces was 0.58, which was neither high nor low. In spite of the significant correlation, the fecal MMP-7 mRNA assay was positive in two cancer patients with negative fecal COX-2 mRNA assay.

In the present study, six patients with CRC had negative fecal RNA test results. Because all of them had positive fecal CEA assay results, the expression levels of which were similar to those of positive fecal COX-2 mRNA assay samples, negative fecal RNA test seems not to be caused by the poor quality of fecal RNA. It is possible that the reason for negative fecal RNA test may be due to tumor biology; that is, the tumors do not express these markers and therefore they would not be detectable in the tissue. In that case, we may increase the sensitivity by adding other tumor-specific markers. Unfortunately, we did not evaluate the expression of these in resected materials.

Specificity and sensitivity are important factors to select the screening test. Low-sensitivity CRC screening increases the number of colonoscopies required, thereby increasing the cost and the risk of complications such as perforation of the colon. In the present study, fecal RNA test using COX-2 and MMP-7 mRNAs showed high specificity due to optimization of PCR. However, it is needed to analyze a much broader spectrum of controls to monitor specificity for these assays.

In this study, we performed fecal RNA test on all patients using stool samples at between 2 and 4 weeks after colonoscopy. It is not certain whether colonoscopy together with forceps biopsy influences on the results of fecal COX-2 mRNA and MMP-7 mRNA expression. Further evaluation is needed to clarify this issue.

In summary, this study showed that it is possible to detect MMP-7 mRNA as well as COX-2 mRNA in feces of CRC patients irrespective of clinical stage. By adding MMP-7 mRNA to fecal COX-2 mRNA assay, the sensitivity of the test increased to 90% for CRC that is significantly higher than that of a single IFOBT. Modifying fecal RNA test by adding appropriate biomarkers might provide higher sensitivity. The value of fecal RNA test, a combination of the fecal COX-2 mRNA and MMP-7 mRNA assays as a screening test for CRC, however, remains unclear due to the limitations of this study. The fecal COX-2 mRNA and MMP-7 mRNA assays for controls were done unblinded to clinical information, and with small sample numbers especially of controls. In monitoring the specificity for a much broader spectrum of controls, including normal healthy controls, a prospective study to compare fecal RNA test using COX-2 and MMP-7 mRNAs with IFOBT is needed. Once the above-mentioned issues are solved, fecal RNA test using COX-2 and MMP-7 mRNAs would be attractive for CRC screening.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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