Detection of fecal interferon-induced transmembrane protein messenger RNA for colorectal cancer screening

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Abstract. Interferon-induced transmembrane protein (IFITM) is reported to be frequently overexpressed in colorectal tumors. This study aimed to determine the usefulness of detecting fecal IFITM messenger RNA (mRNA) by real-time reverse-transcription polymerase chain reaction (RT-PCR) for colorectal cancer (CRC) screening. This pilot study included 21 patients with CRC and 23 healthy controls. Total RNA was isolated from the feces of the patients, and the expression levels of the mRNA of IFITM1, IFITM2 and IFITM3 were measured by real-time RT-PCR to detect CRC. Receiver operating characteristic curves of respective genes were generated, and the area under the curve (AUC), sensitivity and specificity were determined. When the 44 patients were analyzed, the AUCs of fecal IFITM1, IFITM2 and IFITM3 expression analysis were 0.82, 0.80 and 0.65, respectively. The sensitivities were 67% [14/21; 95% confidence interval (CI) 43-85%], 67% (14/21; 95% CI 43-85%) and 71% (15/21; 95% CI 48-89%), respectively; and the specificities were 96% (1/23; 95% CI 78-100%), 96% (1/23; 95% CI 78-100%) and 61% (9/23; 95% CI 39-80%), respectively. When IFITM1 and IFITM2 were combined, the sensitivity was 86% (18/21; 95% CI 64-97%) and the specificity was 96% (1/23; 95% CI 78-100%). The fecal expression analysis of IFITM1 and IFITM2 mRNA by real-time RT-PCR for CRC screening exhibited high specificities, and the sensitivity was further improved by combining IFITM1 and IFITM2.

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Abbreviations: ACTB, β-actin; AUC, area under the curve; CI, confidence interval; CRC, colorectal cancer; Ct, cycle threshold; FOBT, fecal occult blood testing; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; IFITM, interferon-induced transmembrane protein; mRNA, messenger RNA; PCR, polymerase chain reaction; ROC, receiver operating characteristic; RT, reverse transcription

Key words: colorectal cancer, feces, interferon-induced transmembrane protein, screening, RNA

Introduction

Colorectal cancer (CRC) is one of the most common types of cancer. Approximately 1 million new cases are diagnosed and approximately 529,000 patients succumb to this type of cancer worldwide each year (1). When this cancer is diagnosed with localized disease, the five-year survival rate following curative surgery is approximately 90% (2). However, the prognosis worsens with advancing stage, and only 5% of patients diagnosed with distant metastasis survive for five years. Detection of CRC in the early stage is therefore a key factor for reducing CRC mortality rates.

Among the various screening tests for CRC, fecal occult blood testing (FOBT) is considered to be the most effective non-invasive screening test. FOBT is convenient and relatively cost-effective (3-6). Guaiac-based FOBT reduces incidence and mortality (3,7,8), but does not exhibit high sensitivity (9-11). Immunochemical FOBT was reported to have a sensitivity of 65.8% and a specificity of 95% for detecting CRC (12). Immunochemical FOBT exhibits a higher sensitivity than that of guaiac-based FOBT. However, improvement in the sensitivity of the fecal test for CRC screening is required to reduce mortality rates from this type of cancer.

Numerous screening methods for CRC using fecal DNA are available. Methods using fecal DNA allow for the detection of mutated (13-16), methylated (17-22) or long DNA (21,23,24). Results from various studies on fecal RNA-based analysis by reverse-transcription polymerase chain reaction (RT-PCR) for CRC screening have been reported (25-31). Altered messenger RNA (mRNA) expression of numerous genes has been noted in CRC, but only a few genes have been studied to investigate the usefulness of fecal RNA analysis in CRC detection.

Interferon-induced transmembrane protein (IFITM) mRNA has been found to be overexpressed in CRC tissues compared with expression levels in normal tissues by cDNA microarrays (32). Three homologues (IFITM1, IFITM2 and IFITM3) of the human IFITM gene exist. Frequent up-regulation of the IFITM gene expression has been reported to be highly specific to human colorectal carcinogenesis (33).

Quantification by real-time PCR is considered to be useful for determining the optimal cut-off point for discriminating between patients with and without CRC. However, the usefulness of detecting fecal mRNA by real-time RT-PCR for CRC

Table I. The characteristics of the CRC and control patients.

	CRC patients	Control patients
No.	21	23
Gender (male/female)	14/7	14/9
Median age (range)	74 (56-94)	61 (40-80)

screening has yet to be studied sufficiently (29). The usefulness of detecting the fecal mRNA of IFITM1, IFITM2 and IFITM3 by real-time RT-PCR for CRC screening was therefore examined.

Materials and methods

Study design. This study consisted of 21 CRC and 23 control patients (Table I), all of whom underwent colonoscopy. The reasons for performing colonoscopy in the CRC and control patients included positive results of a FOBT test, abdominal pain, anemia, constipation and CRC screening. Stool samples of CRC patients diagnosed with both colonoscopy and histologically were collected prior to surgical resection. The median age of the patients with CRC was 74 years (range 56-94). There were 14 male and 7 female CRC patients. The primary tumor sites were: rectum, 5 patients; sigmoid colon, 7 patients; descending colon, 0 patients; transverse colon, 2 patients; ascending colon, 4 patients; and cecum, 3 patients. The median size of the primary tumors of 14 patients with CRC was 34 mm (range 10-70) and the size of the tumors of the remaining 7 patients with CRC was unknown. The tumors were classified according to Dukes' staging, yielding stage A (n=9), and stages B (n=3), C (n=7) and D (n=2) (Table II). A total of 23 control patients (14 male and 9 female) who did not exhibit neoplastic lesions colonoscopically were also included in this study. The median age of the control patients was 61 years (range 40-80). This study was approved by the ethics committees at the institutions in which fecal samples were collected. Oral and written informed consent was obtained from the patients.

Fecal sample collection and RNA isolation. Fecal samples were initially preserved at -80˚C within 24 h following evacuation. Total RNA was extracted from 500 mg of feces without isolating colonocytes using a combination of Isogene (Nippon Gene, Toyama, Japan) and RNeasy kit (Qiagen, Tokyo, Japan) as previously described (26).

cDNA synthesis. The concentration of isolated RNA was measured by NanoDrop 1000 (Thermo Fisher Scientific, Yokohama, Japan). cDNA was synthesized using SuperScript III RNase H^- reverse transcriptase (Invitrogen, Tokyo, Japan) with 1000 ng fecal total RNA and 150 ng random primers in a total reaction volume of 18 μl according to the manufacturer's instructions.

Table II. The characteristics of the CRC patients.

Patient no.	Age (years)	Gender (male/female)	Location	Size (cm)	Dukes' stage
1	63	M	R	un	C
2	94	M	R	un	B
3	57	M	S	3.3	B
4	74	F	Ce	un	D
5	75	M	R	5.5	A
6	56	M	S	un	A
7	71	M	R	4.0	C
8	60	M	R	7.0	A
9	70	F	S	1.3	A
10	85	M	T	un	C
11	75	M	Ce	un	C
12	85	M	A	un	C
13	86	M	Ce	1.0	A
14	82	F	S	1.5	A
15	81	M	A	4.0	A
16	70	M	S	1.5	A
17	70	M	S	2.0	A
18	65	F	A	4.0	C
19	72	F	T	2.5	C
20	88	F	S	3.5	B
21	80	F	A	4.0	D

Ce, cecum; A, ascending; T, transverse; D, descending; S, sigmoid; R, rectum; un, unknown.

Table III. The primer and probe oligo sequences.

Gene	
GAPDH	Forward: GAACGGGAAGCTTGTCATCA
GAPDH	Reverse: ATCGCCCCACTTGATTTTG
GAPDH	Probe: FAM-CCCATCACCATCTTCCAGGAGCGAGA-TAMRA
ACTB	Forward: CCTCGCCTTTGCCGATCC
ACTB	Reverse: CATGCCGGAGCCGTTGTC
ACTB	Probe: FAM-CGTCCACACCCGCCGCCAGC-TAMRA
IFITM1	Forward: TCGCCTACTCCGTGAAGTCT
IFITM1	Reverse: TGTCACAGAGCCGAATACCA
IFITM1	Probe: FAM-ATGCCTCCACCGCCAAGTGCCT-TAMRA
IFITM2	Forward: TGTATCCCACGTACTCTATCTTCC
IFITM2	Reverse: GGACAGGGCGAGGAATGG
IFITM2	Probe: FAM-TGGAGTAAGTGGAATACAGGTCAAGGGCAG-TAMRA
IFITM3	Forward: CTGAGAACCATCCCAGTAACCC
IFITM3	Reverse: ACTGTTGACAGGAGAGAAGAAGG
IFITM3	Probe: FAM-CATGGTGTCCAGCGAAGACCAGCGG-TAMRA

GAPDH, glyceraldehyde-3-phosphate dehydrogenase; ACTB, β-actin; IFITM, interferon-induced transmembrane protein; FAM, carboxy-fluorescein-aminohexyl amidite; TAMRA, tetramethylrhodamine.

Real-time polymerase chain reaction. Amplification and detection were performed by real-time PCR with a Taq Man probe. The expression levels of glyceraldehyde-3-phosphate dehydrogenase (GAPDH), β-actin (ACTB), IFITM1, IFITM2 and IFITM3 were measured. The sequences of the PCR primers and probes are listed in Table III. cDNA (2 μl) was used as templates in each reaction. The reaction mixture consisted of templates, 10 μl of QuantiTect Multiplex PCR kit (Qiagen, Tokyo, Japan), 0.40 μM of forward and reverse primers and 0.20 μM of the probe in a total reaction volume of 20 μl. The real-time PCR reaction was performed with precycling heat activation at 95˚C for 10 min, followed by 50 cycles of denaturation at 95˚C for 15 sec and annealing/extension at 60˚C for 60 sec in an Applied Biosystems 7500 sequence detection system (Life Technologies, Tokyo, Japan).

Statistical analysis. In the fecal RNA analysis used for detecting CRC, when an amplification curve crossed the threshold line within the end of definite cycles, the expression of the gene was interpreted as positive, and multiple pairs of sensitivities and specificities were determined for each gene. The receiver operating characteristic (ROC) curve was created from pairs of sensitivities and specificities, and the area under the curve (AUC) was calculated (34). The optimal sensitivity and specificity were determined using the Youden index: (Youden index) = maximum (sensitivity + specificity – 1). The sensitivities and specificities were estimated relative to the results of the colonoscopy in the usual manner; 95% confidence interval (CI) for each of the estimated parameters was based on the exact binominal distribution. P<0.05 was considered to be statistically significant. The reported P-values were evaluated by a two-sided test.

Results

RNA concentration. The mean concentrations of RNA extracted from the feces of the 21 CRC and 23 control patients were 876 ng/μl (range 105-3,333) and 1,044 ng/μl (range 105-2,000), respectively. No significant difference was found between RNA concentrations in the group of CRC patients and the group of control patients (P=0.40).

Detection of GAPDH and ACTB mRNA. The detection rates of GAPDH were 91% (19/21) in the CRC patients and 100% (23/23) in the control patients. No significant difference was noted between the detection rates of GAPDH in the CRC and control patients (P=0.2). The cycle threshold (Ct) values of GAPDH in the CRC patients were significantly smaller than those in the control patients (P=0.02). The detection rates of ACTB were 76% (16/21) in the CRC patients and 91% (21/23) in the control patients. No significant difference was found between the detection rates of ACTB in the CRC and control patients (P=0.23). The Ct values of ACTB in CRC patients were significantly smaller than those in the control patients (P=0.003).

Sensitivities and specificities of the gene expression analysis. When the 44 cases were analyzed, the AUCs of fecal IFITM1, IFITM2 and IFITM3 expression analysis for CRC were 0.82, 0.80 and 0.65, respectively (Fig. 1). The sensitivities were 67% (14/21; 95% CI 43-85%), 67% (14/21; 95% CI 43-85%) and 71% (15/21; 95% CI 48-89%), respectively. The specificities were 96% (1/23; 95% CI 78-100%), 96% (1/23; 95% CI 78-100%) and 61% (9/23; 95% CI 39-80%), respectively. The Youden index values were 0.62, 0.62 and 0.32, respectively (Table IV).

Table IV. The sensitivities and specificities of fecal RNA expression analysis.

Expression	Gene	Sensitivity No.	Sensitivity % (95% CI)	Specificity No.	Specificity % (95% CI)	Threshold (cycles)	Youden index
ALL							
	IFITM1	14/21	67 (43-85)	1/23	96 (78-100)	45	0.62
	IFITM2	14/21	67 (43-85)	1/23	96 (78-100)	39	0.62
	IFITM3	15/21	71 (48-89)	9/23	61 (39-80)	38	0.32
	IFITM1+2	18/21	86 (64-97)	1/23	96 (78-100)	IFITM1:45 IFITM2:35	0.81
GAPDH (+)							
	IFITM1	14/19	74 (49-91)	1/23	96 (78-100)	45	0.69
	IFITM2	14/19	74 (49-91)	1/23	96 (78-100)	39	0.69
	IFITM3	15/19	79 (54-94)	9/23	61 (39-80)	38	0.40
	IFITM1+2	18/19	95 (74-100)	1/23	96 (78-100)	IFITM1:45 IFITM2:35	0.90
ACTB (+)							
	IFITM1	12/16	75 (48-93)	1/21	95 (76-100)	44	0.70
	IFITM2	14/16	88 (62-99)	1/21	95 (76-100)	39	0.83
	IFITM3	14/16	88 (62-99)	7/21	67 (43-85)	38	0.54
	IFITM1+2	16/16	100 (79-100)	0/21	100 (84-100)	IFITM1:44 IFITM2:35	1.00

No., the number of positive/total; +, positive gene expression. ALL, all 44 cases.

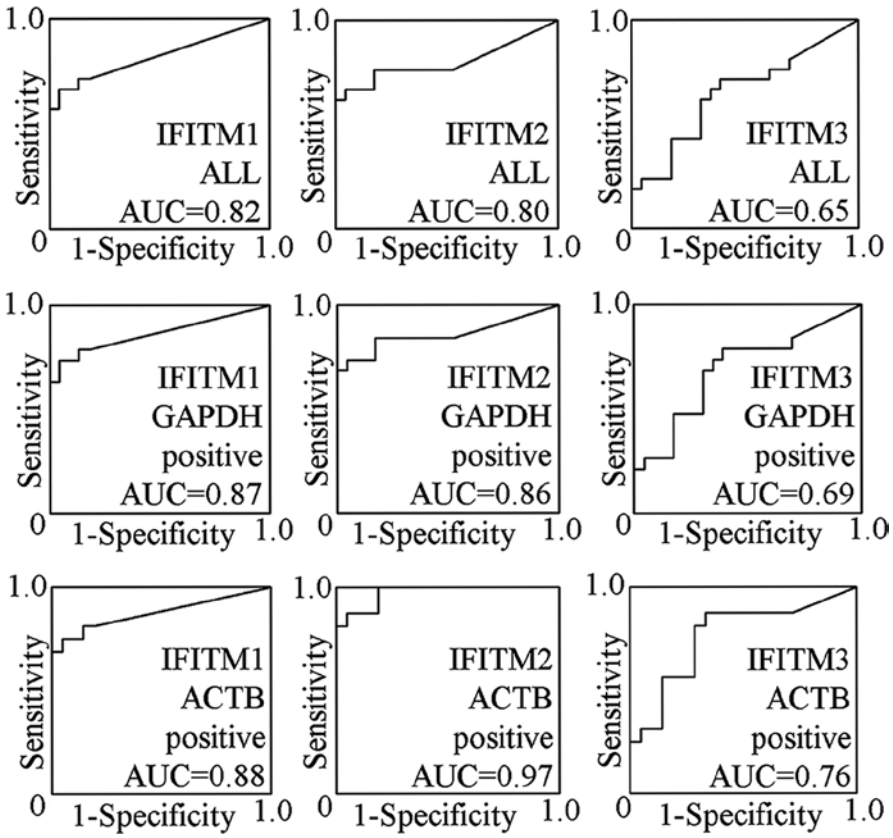

Figure 1. ROC curves and AUC of fecal RNA expression analysis. The markers were determined in all 44 cases (21 CRC and 23 control patients), in the cases whose fecal mRNA expression of GAPDH was positive (19 CRC and 23 control patients) and in the cases whose fecal mRNA expression of ACTB was positive (16 CRC and 21 control patients). ALL, all 44 cases.

When 42 cases were analyzed in which GAPDH mRNA was detected, the AUCs of fecal IFITM1, IFITM2 and IFITM3 expression analysis for CRC were 0.87, 0.86 and 0.69, respectively (Fig. 1). The sensitivities were 74% (14/19; 95% CI 49-91%), 74% (14/19; 95% CI 49-91%) and 79% (15/19; 95% CI 54-94%), respectively. The specificities were 96% (1/23; 95% CI 78-100%), 96% (1/23; 95% CI 78-100%) and 61% (9/23; 95% CI 39-80%), respectively, and the Youden index values were 0.69, 0.69 and 0.40, respectively (Table IV).

When 37 cases were analyzed in which ACTB mRNA was detected, the AUCs of fecal IFITM1, IFITM2 and IFITM3 expression analysis for CRC were 0.88, 0.97 and 0.76, respectively (Fig. 1). The sensitivities were 75% (12/16; 95% CI 48-93%), 88% (14/16; 95% CI 62-99%) and 88% (14/16; 95% CI 62-99%), respectively. The specificities were 95% (1/21; 95% CI 76-100%), 95% (1/21; 95% CI 76-100%) and 67% (7/21; 95% CI 43-85%), respectively, and the Youden index values were 0.70, 0.83 and 0.54, respectively (Table IV).

The sensitivities of fecal IFITM1, IFITM2 and IFITM3 expression analysis of the patients with Dukes' A+B were 58% (7/12; 95% CI 30-87%), 67% (8/12; 95% CI 38-95%) and 67% (8/12; 95% CI 38-95%), respectively, and the patients with Dukes' C+D exhibited 78% (7/9; 95% CI 45-100%), 67% (6/9; 95% CI 34-99%) and 78% (7/9; 95% CI 45-100%), respectively. Therefore, no significant difference was found in the sensitivities between the two groups. There was also no significant difference in the sensitivities of fecal IFITM1, IFITM2 and IFITM3 expression analysis regarding gender, tumor location, tumor size, RNA concentration or GAPDH expression. The sensitivities of fecal IFITM2 and IFITM3 expression analysis of the group in which ACTB expression was positive were significantly higher than those of the group in which ACTB expression was negative (P=0.001 and P=0.01, respectively), although no significant difference was found in the sensitivity of fecal IFITM1 expression analysis between the two groups.

Combination of IFITM1 and IFITM2. The AUCs of IFITM1 and IFITM2 were larger than that of IFITM3. Therefore, we calculated the sensitivities and specificities for the combination of IFITM1 and IFITM2 (fecal IFITM1+2 expression analysis). When analyzed for all 44 cases, the sensitivity and specificity of fecal IFITM1+2 expression analysis were found to be 86% (18/21; 95% CI 64-97%) and 96% (1/23; 95% CI 78-100%). When analyzed for cases in which GAPDH mRNA was detected, the sensitivity and specificity were 95% (18/19; 95% CI 74-100%) and 96% (1/23; 95% CI 78-100%). When analyzed for cases in which ACTB mRNA was detected, the sensitivity and specificity were 100% (16/16; 95% CI 79-100%) and 100% (0/21; 95% CI 84-100%) (Table IV).

The sensitivity of fecal IFITM1+2 analysis of patients with Dukes' A+B and that of patients with Dukes' C+D were 83% (10/12; 95% CI 55-100%) and 89% (8/9; 95% CI 56-100%), respectively, and no significant difference was noted in the sensitivities between the two groups. There was also no significant difference in the sensitivities of fecal IFITM1+2 expression analysis with regards to gender, tumor location, tumor size or RNA concentration. The sensitivity of fecal IFITM1+2 expression analysis of the group in which GAPDH expression was positive was significantly higher than that of the group in which GAPDH expression was negative (P=0.01), and the sensitivity of the group in which ACTB expression was positive was significantly higher than that of the group in which ACTB expression was negative (P=0.01).

Discussion

Results of numerous studies on fecal DNA-based analysis for CRC screening have been reported. A fecal DNA panel consisting of 21 mutations exhibited 51.6% sensitivity and 94.4% specificity (11). Since CRC cells undergo diverse genetic changes, it is difficult to detect CRC with a high sensitivity by using only fecal DNA-based mutational analysis.

Fecal methylation analysis of the vimentin gene provided sensitivity and specificity of 72.5 and 86.9%, respectively, and the combination of vimentin methylation plus a DNA integrity assay resulted in 87.5% sensitivity and 82% specificity (21). In another study, a fecal SFRP2 methylation assay for CRC screening exhibited 77-90% sensitivity and 77% specificity (20). In the fecal methylation analysis, it is crucial to decrease the effect of methylation with aging to improve the specificity.

The number of studies on fecal RNA-based analysis for CRC screening that are currently available are fewer than those on fecal DNA-based analysis. The fecal RNA expression analysis of PTGS2 by semi-quantitative RT-PCR was reported to have sensitivities of 50-90% and specificities of 93-100% in previous studies (22,26,30). Semi-quantitative RT-PCR was used in the majority of previous studies on fecal RNA expression analysis for CRC screening, while real-time RT-PCR was used in only a few studies (29). Real-time PCR has a number of advantages over semi-quantitative PCR, including high speed, reduction of contamination and a high level of reproducibility, from the viewpoint of a laboratory test. In the present study, the sensitivity and specificity of fecal IFITM1+2 expression analysis determined in all 44 cases were 86 and 96%, respectively. We showed that CRC is potentially detected with a high sensitivity and specificity by fecal RNA expression analysis using real-time RT-PCR.

The quantitation of templates by real-time RT-PCR allowed us to generate ROC curves for the fecal mRNA expression analysis, compare the AUCs, and determine the cut-off points at which optimal sensitivities and specificities are achieved. Real-time RT-PCR is considered to be effective in determining the optimal cut-off points efficiently when studying the usefulness of fecal mRNA expression analysis by RT-PCR for CRC screening.

Up-regulation of the IFITM gene was considered to be an early event in β-catenin intestinal tumorigenesis (33). No significant difference was noted in the sensitivities of fecal IFITM1, IFITM2, IFITM3 and IFITM1+2 expression analysis between the group of CRC patients without metastasis (Dukes' A+B) and the group of CRC patients with metastasis (Dukes' C+D). Therefore, the fecal IFITM expression analysis appears to be useful in the early detection of CRC.

No significant difference was found in sensitivities regarding tumor size and location. However, the sensitivity for tumors of less than 34 mm in diameter were lower than that for tumors of more than 34 mm in diameter. The sensitivity for tumors located on the right side of the colon were lower than that for tumors located on the left side of the colon. A larger study is therefore required to clarify the differences in

sensitivities of fecal mRNA expression analysis for CRC with regards to tumor size and/or location.

When analyzed for cases in which the mRNA of the housekeeping gene was detected, AUCs were found to be larger than AUCs when analyzed for all 44 cases. Moreover, the sensitivity of fecal IFITM1+2 expression analysis in the group in which the mRNA of the housekeeping gene was not detected was significantly lower than that in the group in which the mRNA of the housekeeping gene was detected. Fecal IFITM mRNA-negative CRC cases appear to include not only cases in which IFITM mRNA was not expressed in their tumors but also ones in which human RNA as templates of RT-PCR was insufficient in their fecal samples.

In conclusion, detection of fecal mRNA of IFITM1 and IFITM2 had larger AUCs than that of IFITM3, and the sensitivity was improved by combining IFITM1 and IFITM2. Since the number of cases analyzed in the present study was limited, a larger study is imperative in order to assess sensitivity and specificity. However, the results of the present study suggest the usefulness of detecting the fecal mRNA of IFITM1 and IFITM2 by real-time RT-PCR for CRC screening.

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