An invited review following *the Soujinkai Young Investigator Award*: Multitarget Stool Test for Detecting Advanced Colorectal Adenomas

Running title: A Novel Index for Advanced Colorectal Adenoma

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Abstract

The fecal immunochemical test for hemoglobin (FIT) is widely used for colorectal cancer screening but shows limited sensitivity for detecting advanced colorectal adenomas. To overcome this limitation, we evaluated a combined approach using FIT and fecal DNA testing for methylated somatostatin (*SST*). Fecal samples were collected from healthy subjects, patients with non-advanced adenoma, advanced adenoma, and colorectal cancer. *SST* methylation levels were quantified using droplet digital PCR after treatment with methylation-sensitive restriction enzymes. Using logistic multivariate analysis, we developed the FAMS (FIT, age, methylated *SST*) index. The FAMS index improved sensitivity for detecting advanced adenoma compared with FIT alone while maintaining high specificity. Even at higher specificity, the sensitivity of the FAMS index remained higher than that of FIT. These results suggest that the FAMS index can non-invasively enhance early detection of advanced colorectal adenoma and may serve as a promising tool for screening.

Key words: advanced colorectal adenoma, fecal DNA test, fecal immunochemical test for hemoglobin, methylation, SST

Introduction

Colorectal cancer (CRC) is the third most commonly diagnosed cancer and the second leading cause of cancer deaths worldwide. In 2020, an estimated 1.9 million new cases were reported, with 935,000 deaths, representing roughly one in ten cancers and cancer-related deaths globally.[1] Early detection is crucial, as surgery at an early stage can cure over 95% of patients, including those with premalignant lesions.[2] Among screening methods, the fecal immunochemical test for hemoglobin (FIT) is widely used and effective for CRC,[3] but it has limited sensitivity for advanced colorectal adenomas (AA), which are considered precancerous

lesions.[4] The reported sensitivity of FIT for AA is only about 23.8%–27.1%, compared with 65.8%–73.8% for CRC.[5, 6]

To overcome this limitation, we developed the combined restriction digital PCR (CORD) assay, a highly sensitive method capable of detecting even a single copy of a methylated gene from a limited amount of DNA sample.[7, 8] Among the genes we examined, somatostatin (*SST*) was found to be hypermethylated in colorectal neoplastic tissues (unpublished data). This suggested that methylated *SST* could serve as a biomarker for colorectal neoplasia. We therefore investigated whether measuring *SST* methylation in fecal samples using the CORD assay in combination with FIT could improve the detection of AA.

Clinical materials

We prospectively enrolled 377 participants from St. Hill Hospital, Ajisu Kyoritsu Hospital, IMSUT Hospital, and Yamaguchi University Hospital between October 2007 and December 2019. Of these, 365 participants had complete evaluable results, including 79 individuals with negative colonoscopy findings (control group), 43 cases of non-advanced colorectal adenomas (NAA), 117 cases of AA, and 126 cases of CRC. AA was defined as adenomas of 1 cm or larger in size, or with high-grade or severe dysplasia, or with villous components (tubulovillous or villous).[5, 9, 10] Non-neoplastic polyps and non-advanced adenomatous polyps not classified as AA were grouped as NAA.[5] Staging followed the criteria of the Union for International Cancer Control (International Union Against Cancer).[11]

Performance of FIT in detecting advanced colorectal adenoma

We used a cutoff value of 20 μg hemoglobin/g feces, equivalent to 100 ng hemoglobin/mL buffer.[12, 13] The sensitivity of FIT was 7.0% (3/43) for NAA, 29.1% (34/117) for AA, and 91.3%

(115/126) for CRC. The specificity was 87.3% (69/79) for the control group and 89.3% (109/122) for the control/NAA group (Table 1).

Assessment of *SST* methylation in fecal DNA for advanced colorectal adenoma detection

The fecal DNA test comprised two evaluation metrics: the absolute copy number of methylated SST and the methylation ratio, calculated by dividing the methylated SST copy number by the hTERT copy number. Since human DNA content in fecal samples can vary with stool consistency (e.g., watery versus solid), the methylation ratio was included to enable assessment across all stool forms. The distributions of the methylated *SST* copy number and the methylated *SST* ratio are shown in Fig. 1A and C, respectively. The methylated SST copy number was significantly higher in the AA group than in the control group (Fig. 1A), and also significantly higher in the CRC group than in the control group and in the NAA group (Fig. 1A). Notably, the SST methylation ratio was significantly elevated in the AA group compared to all other groups (Fig. 1C).

We set 67.9 copies/test as the cutoff for the methylated *SST* copy number (Fig. 1A) and 0.50 for the *SST* methylation ratio to achieve approximately 95% specificity (Fig. 1C). Fecal DNA test was considered positive if either or both parameters exceeded the respective cutoffs. Using these criteria, we found a sensitivity of 14.0% (6/43) for NAA, 39.3% (46/117) for AA, and 30.2% (38/126) for CRC with a specificity of 92.4% (73/79) for the control group and 90.2% (110/122) for the control/NAA group (Table 1).

Combined use of FIT and fecal DNA test for enhanced detection of advanced colorectal adenoma

The combination of FIT and fecal DNA test (combination test) was defined as positive if either FIT or the fecal DNA test was positive, or if both were positive. The sensitivity of the combination test was 20.9% (9/43) for NAA, 61.5% (72/117) for AA, and 94.4% (119/126) for CRC, with a specificity of 79.7% (63/79) for the control group and 79.5% (97/122) for the control/NAA group (Table 1). Among 117 patients with AA, 72 were positive on the combination test: 26 for FIT alone, 38 for fecal DNA test alone, and 8 for both tests (Fig. 2). Of the 38 patients positive only on fecal DNA test, 14 were positive for methylated SST copy number alone, 19 for SST methylation ratio alone, and 5 for both (Fig. 2).

Interestingly, FIT and the fecal DNA test complemented each other. The sensitivity for advanced adenomas was 29.1% for FIT alone and 39.3% for the fecal DNA test alone, while the combination test improved sensitivity to 61.5% (Table 1). These findings indicate that the combination test effectively enhances AA detection.

Development and performance of the FAMS index for predicting advanced colorectal adenoma

While the combination test achieved moderate sensitivity (61.5%) for AA, the specificity of approximately 80% remained suboptimal for clinical application (Table 1). To address this issue, we conducted multiple logistic regression analyses using variables (fecal DNA test, FIT, age, and sex) to identify independent factors associated with AA/CRC. As a result, fecal DNA test, FIT, and age were the independent predictors (Table 2). Using these variables, we established a prediction formula as shown below and named it the FAMS (FIT, age, methylated *SST*) index.

$$FAMS\ index = \frac{1}{1 + e^{-X}}$$

$$X = -6.441 + 1.622 \times [Fecal\ DNA\ test] + 2.69 \times [FIT] + 0.09609 \times Age$$

In this formula, both [Fecal DNA test] and [FIT] are treated as binary variables, with a value of 1 assigned if the test result is positive and 0 if negative. Age is a continuous variable (years). The FAMS index, ranging from 0 to 1, quantitatively reflects the probability of AA/CRC, with smaller values indicating lower risk and larger values indicating higher risk. The distributions of the FAMS index across the groups are shown in Fig. 3A, with the AA group exhibiting significantly higher values than both the control and NAA groups ($P < 0.0001$). Furthermore, the CRC group had significantly higher FAMS index values compared to all other groups ($P < 0.0001$).

Subsequently, we performed ROC analyses. The area under the ROC curve (AUC) was 0.90 for distinguishing the control/NAA group from the AA/CRC group (Fig. 3B) and 0.85 for differentiating the control/NAA group from the AA group (Fig. 3C). At the specificity points of 80.3%, 86.1%, 91.0%, and 94.3%, the sensitivities for AA were 68.4%, 61.5%, 56.4%, and 42.7%, respectively (Table 3).

In comparison with other diagnostic tests, the FAMS index demonstrated a sensitivity of 56.4% for detecting AA at a specificity of 91.0% (Table 3), which is approximately twice that of FIT alone (sensitivity 29.1%, specificity 89.3%; Table 1). Moreover, the FAMS index showed superior performance over the combination test, achieving a sensitivity of 68.4% compared with 61.5% for the combination test at a similar specificity of about 80% (Tables 1 and 3).

Conclusion

Our study demonstrates that combining FIT with fecal DNA test based on *SST* methylation improves the detection of advanced adenomas, which are otherwise poorly identified by FIT alone. Moreover, the FAMS index, combining fecal DNA test, FIT, and age, further enhances

diagnostic accuracy. This approach requires only a small fecal sample, making it practical for clinical implementation. Further validation in larger cohorts is needed to confirm these findings and establish the utility of this method in routine screening.

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All figures and tables in this manuscript are reproduced or adapted from our previously published work[14] in accordance with the publisher's policy.

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Conflict of interest

Yutaka Suehiro and Takahiro Yamasaki received grants from Eiken Chemical. The other authors declare no conflict of interest.

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Figure legends

Fig. 1 Fecal DNA test. Distributions of methylated *SST* copy numbers (A), *hTERT* copy numbers (B), and ratios of methylated *SST* to *hTERT* copy numbers (C) in each group are shown. The *P* values are only shown for comparisons with *P* value less than 0.05. Each sample is indicated by a closed circle. The solid horizontal lines indicate medians, and the dotted lines indicate cutoff points. AA, advanced colorectal adenoma; CRC, colorectal cancer; NAA, non-advanced colorectal adenoma.

Fig. 2 Venn diagrams. Copy number, number of subjects with copy number of methylated *SST* ≥ 67.9 copies/test; FIT, number of subjects with positive fecal immunochemical test for hemoglobin; Ratio, number of subjects with the ratio of methylated *SST* to *hTERT* copy numbers ≥ 0.5.

Fig. 3 Diagnostic performance of the FAMS index. The distribution of the FAMS index in each group is shown (A). Each sample is indicated by a closed circle. The solid horizontal lines indicate medians. Receiver operating characteristic curve analyses between the control/NAA group and the AA/CRC group (B), and between the control/NAA group and the AA group (C) are shown. AA, advanced colorectal adenoma; AUC, area under the ROC curve; CI, confidence interval; CRC, colorectal cancer; FAMS index, multivariate analysis consisting of fecal immunochemical test for hemoglobin, age, and methylated *SST*; NAA, non-advanced colorectal adenoma.

Fig. 1

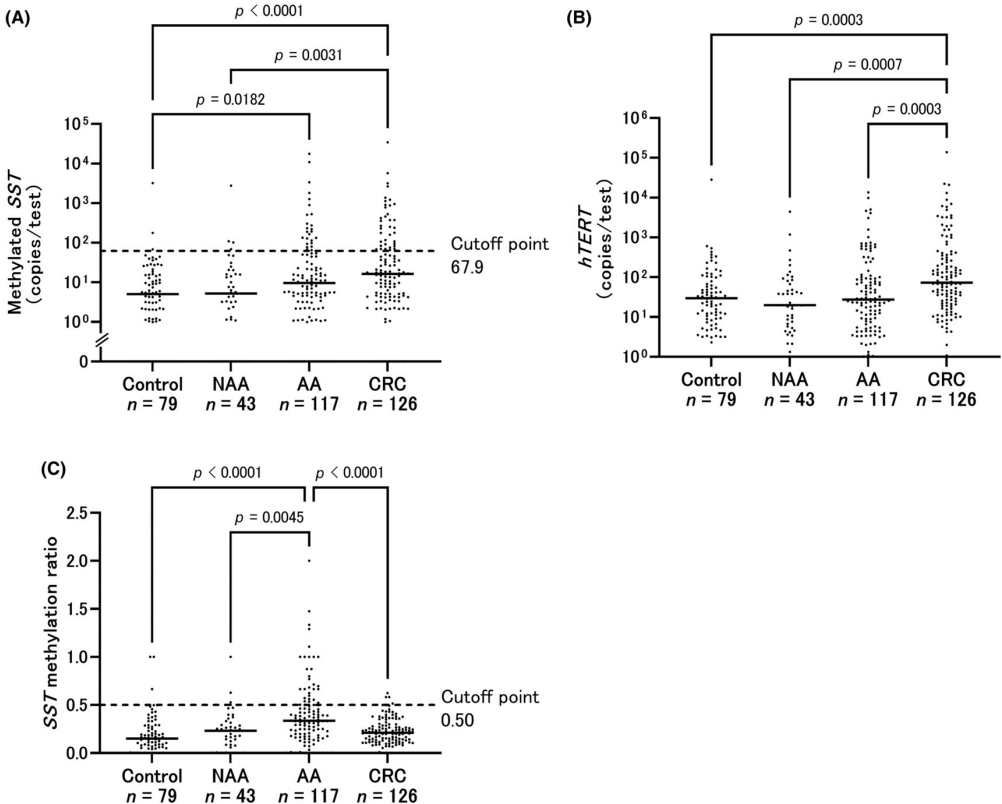

(A)
p < 0.0001
p = 0.0031
p = 0.0182
Methylated SST (copies/test)
Cutoff point 67.9
Control n = 79
NAA n = 43
AA n = 117
CRC n = 126
(B)
p = 0.0003
p = 0.0007
p = 0.0003
hTERT (copies/test)
Control n = 79
NAA n = 43
AA n = 117
CRC n = 126
(C)
p < 0.0001
p < 0.0001
p = 0.0045
SST methylation ratio
Cutoff point 0.50
Control n = 79
NAA n = 43
AA n = 117
CRC n = 126

Fig. 2

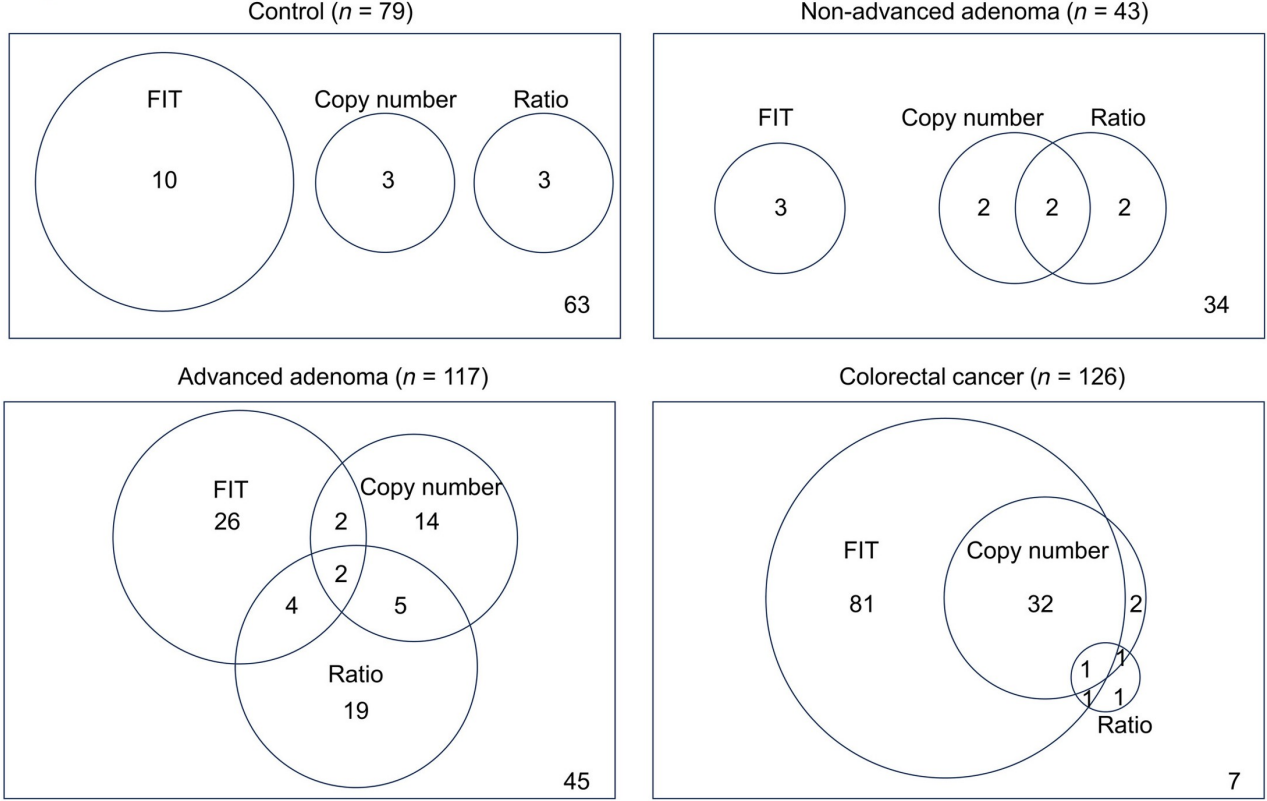
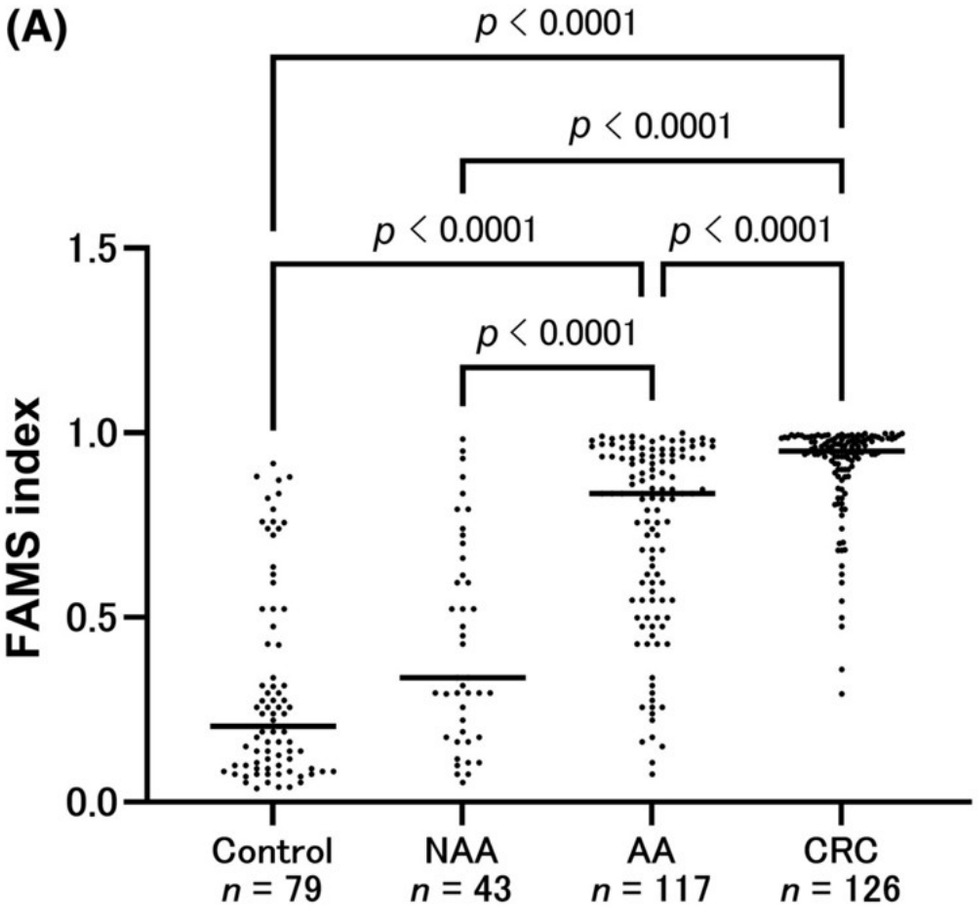
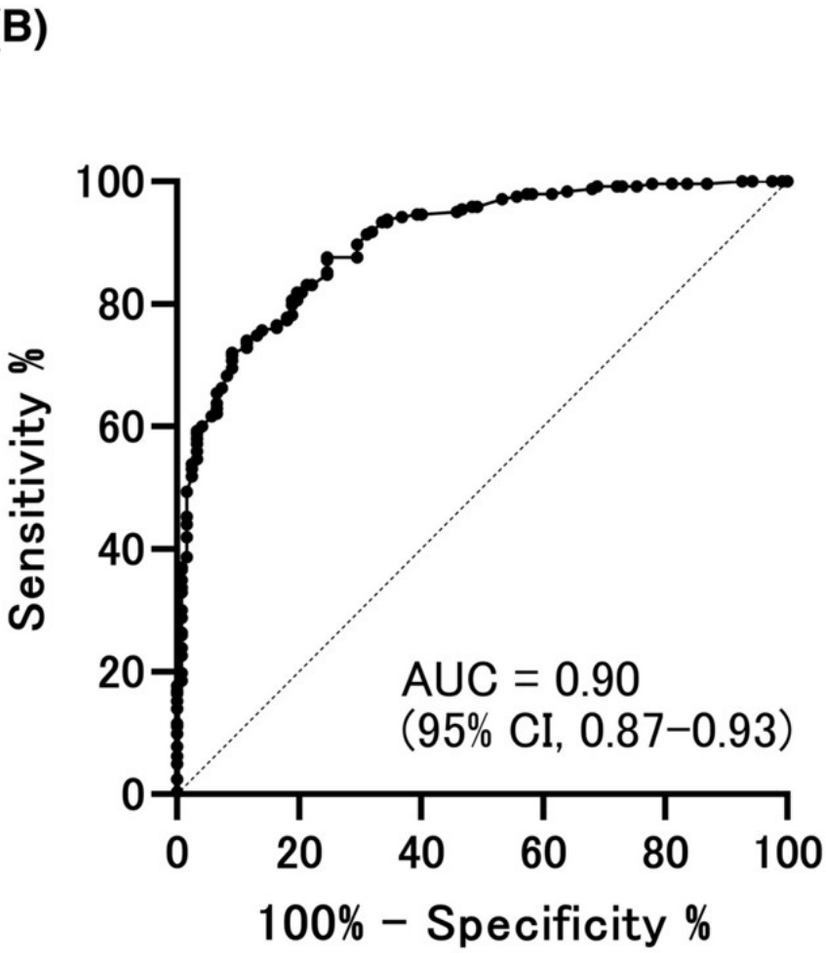

Control (n = 79)
FIT
10
Copy number
3
Ratio
3
63
Non-advanced adenoma (n = 43)
FIT
3
Copy number
2
2
Ratio
2
34
Advanced adenoma (n = 117)
FIT
26
2
Copy number
14
2
4
5
Ratio
19
45
Colorectal cancer (n = 126)
FIT
81
Copy number
32
2
1
1
1
1
Ratio
7

Fig. 3


(A)
p < 0.0001
p < 0.0001
p < 0.0001
p < 0.0001
p < 0.0001
FAMS index
1.5
1.0
0.5
0.0
Control
n = 79
NAA
n = 43
AA
n = 117
CRC
n = 126


(B)
Sensitivity %
100
80
60
40
20
0
AUC = 0.90
(95% CI, 0.87–0.93)
0
20
40
60
80
100
100% – Specificity %

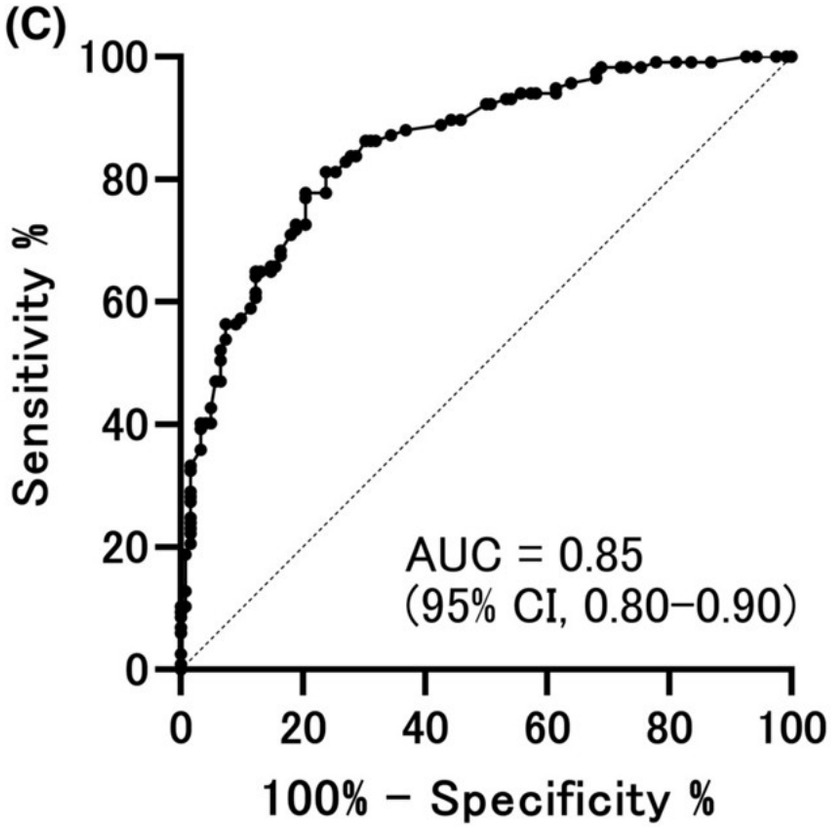

(C)
Sensitivity %
100
80
60
40
20
0
AUC = 0.85
(95% CI, 0.80–0.90)
0
20
40
60
80
100
100% – Specificity %

Table 1. Diagnostic performance of each test for the findings of colonoscopy.

Most advanced finding	(n)	FIT				Fecal DNA test				Combination test			
		Positive results[a] (n)	Sensitivity (%)	95%CI (%)	*P*[b]	Positive results[c] (n)	Sensitivity (%)	95% CI (%)	*P*[b]	Positive results[d] (n)	Sensitivity (%)	95% CI (%)	*P*[b]
NAA	43	3	7.0	2.4–18.6		6	14.0	5.3–27.9		9	20.9	10.0–36.0	
Left	20	2	10.0	1.2–31.7	0.5900	2	10.0	1.2–31.7	0.6688	4	20.0	5.7–43.7	1.0000
Right	23	1	4.3	0.1–21.9		4	17.4	5.0–38.8		5	21.7	7.5–43.7	
AA	117	34	29.1	21.6–37.9		46	39.3	30.5–48.2		72	61.5	52.7–70.4	
Left	46	13	28.3	15.2–41.3	1.0000	14	30.4	17.1–43.7	0.1256	23	50.0	35.6–64.4	0.0518
Right	71	21	29.6	19.0–40.2		32	45.1	33.5–56.6		49	69.0	58.3–79.8	
CRC	126	115	91.3	85.0–95.1		38	30.2	22.1–38.2		119	94.4	88.9–97.7	
Left	84	79	94.0	86.7–98.0	0.1779	31	36.9	26.6–47.2	0.0236	81	96.4	90.0–99.3	0.2206
Right	42	36	85.7	71.5–94.6		7	16.7	7.0–31.4		38	90.5	77.4–97.3	
AA/CRC	243	149	61.3	55.1–67.2		84	34.6	28.6–40.5		191	78.6	73.4–83.8	
Left	130	92	70.8	63.0–78.6	0.0015	45	34.6	26.4–42.8	1.0000	104	80.0	73.1–86.9	0.6388
Right	113	57	50.4	41.2–59.7		39	34.5	25.7–43.3		87	77.0	69.2–84.8	
			Specificity (%)				Specificity (%)				Specificity (%)		
Control	79	10	87.3	78.2–93.0		6	92.4	84.2–97.2		16	79.7	70.9–88.6	
Control/NAA	122	13	89.3	82.6–93.7		12	90.2	84.9–95.4		25	79.5	72.3–86.7	

[a]Criterion for a positive result of FIT is above 20 μg hemoglobin/g feces (100 ng Hb/mL buffer).

[b]*P* value were calculated by Fisher's exact test between left- and right-sided sided tumors.

[c]Criterion for a positive result of fecal DNA testing is either 67.9 or more copy numbers of methylated SST or SST methylation ratio of 0.5 or more or both.

[d]Criterion for a positive result with the combination of FIT and fecal DNA test is either a positive FIT or fecal DNA test or positive for both.

Abbreviations: AA, advanced adenoma; CI, confidence interval; CRC, colorectal cancer; FIT, fecal immunochemical test for hemoglobin; NAA, non-advanced adenoma.