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ORIGINAL ARTICLE

Evaluation of Combined use of Fecal Multigene Mutation Test and Fecal Immunochemical Test for Colorectal Cancer Screening

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SUMMARY

Background: **The aim was to investigate the value of concomitant use of fecal KRAS-APC-p53-BRAF mutation test and a fecal immunochemical test (FIT) for colorectal cancer (CRC) screening.**
Methods: **Stool samples of 279 subjects were collected from the Fujian provincial hospital and divided into five groups: CRC (n = 82); advanced adenoma (AA, n = 76); non-advanced adenoma (NAA, n = 24); healthy control (n = 85); and interference group (n = 12). All stool samples were tested using a fecal multigene mutation (KRAS-APC-p53-BRAF) Kit and FIT.**
Results: **The sensitivity of combined use of fecal multigene mutation test and FIT for detecting CRC [84.15% (69/82)] was significantly higher than that of fecal multigene mutation test [47.56% (39/82), p < 0.001] or FIT [71.95% (59/82), p < 0.001] alone. The sensitivity of combined use for detection of AA [48.68% (37/76)] was also significantly higher than that of multigene mutation test [26.32% (20/76), p < 0.001] or FIT [28.95% (22/76), p < 0.001] alone. The specificity of combined use for detection of NAA and healthy control was 87.16%.**
Conclusions: **The combination of fecal multigene (KRAS-APC-p53-BRAF) mutation test and FIT has greater sensitivity than alone and may be a useful noninvasive method for CRC screening.**
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INTRODUCTION

Colorectal cancer (CRC) is one of the most common malignant tumors in the gastrointestinal tract [1]. In China, the incidence of CRC has shown an increasing trend over successive years. According to the 2019 National Cancer Report, CRC is the third most frequently diagnosed cancer, accounting for 9.88% of malignant tumors [2]. The multistep transition from pre-malignant lesions, advanced adenomas (AA), to CRC may occur over a period of several decades, providing a window of

opportunity for early detection and screening [3,4]. Towards this end, many countries have implemented national CRC screening programs. In the United States, the incidence of CRC and the associated mortality rates have decreased over the last two decades, which is largely attributable to the early diagnosis and treatment of CRC [5]. CRC screening programs have been shown to reduce incidence and mortality.

Currently, the commonly used CRC screening methods include enteroscopy, guaiac-based fecal occult blood test (gFOBT), and fecal immunochemical test (FIT) [6-8]. Although colonoscopy is the standard for CRC screening, the invasive nature of the examination and the requirement for adequate intestinal preparation is a major barrier to its wider use [6]. gFOBT is the most widely used screening method, but its diagnostic accuracy needs to be improved [9-11]. FIT (developed specifically to detect human hemoglobin in stool samples by antibodies) may replace gFOBT as the main test for detection of fecal occult blood. It has a higher sensitivity for detection of CRC and precancerous lesions and is less liable to be affected by diet and drugs [12]. However, use of FIT for CRC screening does not overcome all the problems. Thus, development of more effective strategies for CRC screening is a key goal.

Fecal DNA detection technology is a recently developed molecular marker-based technology for detection of fecal exfoliated cells. An encouraging study showed approximately 70% adherence to fecal DNA tests; in addition, patients with positive fecal DNA test results had a higher colonoscopy completion rate [13]. Studies have shown that fecal DNA detection can be used as a marker for CRC and precancerous lesions [14]. Imperial et al. used a combination of detection of KRAS gene mutation and NDRG4 and BMP3 methylation in fecal DNA [15]. The results showed a high sensitivity of fecal DNA multi-target detection for CRC screening [15]. The US cancer screening guidelines also recommend the application of multi-target fecal detection technology for the screening of CRC [16].

In the present study, we aimed to evaluate the feasibility and accuracy of application of fecal multigene mutation detection combined with FIT for CRC screening. We selected the most frequently mutated genes (KRAS-APC-p53-BRAF) found in CRC for the multigene mutation test. Hot spot mutations of these genes promote the occurrence and development of CRC by interfering with crucial signal pathways (including the Wnt catenin pathway and PI3K pathway) or by hindering DNA repair [17-20]. In this study, we detected 26 loci of the above four genes (KRAS-APC-p53-BRAF) in combination with FIT to explore the feasibility of fecal samples replacing tissue samples and the application of the above gene mutations combined detection kit for early screening of CRC.

MATERIALS AND METHODS

Participants and stool collection

In this study, 279 eligible participants [157 males, 122 females; mean age: 58 years (range 24 - 87)] were recruited at the Department of Gastroenterology of the Fujian Provincial Hospital, including 82 CRC patients, 76 AA patients, 24 NAA patients, 85 healthy controls, and 12 interferences. None of the patients had received any anticancer treatment before admission. Colorectal cancer, advanced adenoma, and non-advanced adenoma were confirmed by postoperative histopathology or colonoscopy. The final diagnosis of patients was determined based on histopathological analysis by an experienced pathologist. The Ethics committee of the Fujian Provincial Hospital approved the study (approval number K2019-11-027). Written informed consent for participation was obtained from all subjects.

Stool samples were collected from 279 participants, then stored in the sample preservation solution in the ratio of 1:4.

FIT analysis

FIT analysis was performed using Immunological Occult Blood Test Kit (W.H.P.M. Inc., USA), following the manufacturer's instructions. In brief, the kit was placed on a clean and flat table; the tip of the collection tube containing the collected fecal samples was broken and the first two drops were discarded. Two drops (approximately 90 µL) of the diluted sample were vertically added in the sampling hole of the kit. Then, we waited for the purplish-red strip to appear; the test result was read in 5 minutes.

Fecal DNA extraction

A DNA extraction kit (MicroDiag Biomedicine, China) was used to extract DNA from fecal samples. Briefly, to enrich DNA, we mixed the collected fecal samples and centrifuged them at 4,500 g for 25 minutes. The supernatant was collected and mixed with PVPP powder in the proportion of 20 mL supernatant plus 1 g PVPP powder, followed by centrifugation at 3,000 g for 15 minutes, and collection of the supernatant again. For targeted DNA regions capture, first the supernatant (10 mL) was mixed with lysis buffer (6.35 mL), 20 × SSC (1.6 mL), and sequence-specific probes. Then, the probes were denatured at 90°C for 10 minutes before hybridization. At room temperature, ten sequence-specific biotin probes targeting different regions were hybridized with nucleic acid templates. Then the target sequences were obtained by streptavidin-modified magnetic beads.

Tissue DNA extraction

DNA was extracted from paraffin-embedded tissue using a paraffin DNA extraction kit (MicroDiag Biomedicine, China). The detailed protocol was as follows. First, a section of paraffin-embedded tissue samples was mixed with 100% xylene (1 mL xylene/4 - 5 paraffin

sections) and was fully dissolved by oscillation. Then, the sample was centrifuged at 12,000 rpm for 2 minutes. After removal of the supernatant, 1 mL anhydrous ethanol was added to each sample and mixed evenly. The sample was centrifuged again at 12,000 rpm for 2 minutes, and the supernatant was discarded. After drying at room temperature, the pellet was resuspended in 400 µL Lysis Buffer FTL. Then, 20 µL of proteinase K was added and vortexed. The sample was incubated at 55°C for 1 hour until it was completely lysed. After additional incubation at 90°C for 1 hour, 300 µL binding buffer BP was added and mixed. The sample was further centrifuged at 12,000 rpm for 2 minutes, and the supernatant was transferred to new tubes. Then, 400 µL anhydrous ethanol was added and gently mixed. The solution was then added to the DNA-adsorbing spin column and centrifuged at 10,000 rpm for 1 minute. The flow-through was discarded, and the DNA-adsorbing spin column was placed back into a collection tube. Then 500 µL washing buffer DWB was added to the spin column and centrifuged at 10,000 rpm for 1 minute. The column was placed at room temperature for 10 - 30 minutes to complete the drying process. Finally, 50 - 100 µL of the solution EB was added to the column, and the column was centrifuged at 10,000 rpm for 1 minute to collect the eluent of DNA.

KRAS-APC-p53-BRAF mutation test

The DNA obtained using the protocol described above was subjected to qPCR. The PCR primers were designed in accordance with the GenBank sequences. The PCR reaction contained 15 µL PCR Mastermix and 15 µL DNA to constitute a final volume of 30 µL. Amplification was performed on an LC480-II PCR thermocycler (Roche Diagnostics). The cycling conditions were as follows: 1 cycle of 5 minutes at 95°C, 45 cycles of 10 seconds at 95°C, 30 seconds at 60°C, and 10 seconds at 40°C. The fecal multigene mutation (KRAS-APC-p53-BRAF) Kit was designed for 26 tumor-specific gene mutation sites (13 KRAS, 5 APC, 2 p53, 6 BRAF). The specific mutated sites of these genes (KRAS-APC-p53-BRAF) are shown in Table 1.

Statistical analyses

All statistical analyses were performed using SPSS 21.0 (IBM SPSS Statistics for Windows; IBM Corp., Armonk, NY, USA) software. Normally-distributed, continuous variables were expressed as mean ± standard deviation, and between-group differences were assessed using the *t*-test. Categorical variables were expressed as frequency (percentage), and between-group differences were assessed using the chi-squared test. Two-sided p-values < 0.05 were considered indicative of statistical significance.

RESULTS

The overall positivity rate of FIT/multigene mutation test alone and combined test

A total of 279 stool samples were analyzed by FIT and fecal multigene mutation (KRAS-APC-p53-BRAF) test. The overall positivity rate of the combined test was 46.24% (129/279), which was higher than that of the FIT or multigene mutation test alone. The positivity rates of combined test in men and women were 49.04% (77/157) and 42.62% (52/112), respectively. The positivity rates in the age-groups of < 40, 40 - 49, 50 - 59, 60 - 69, 70 - 79, and > 79 years were 30.56% (11/36), 40.54% (15/37), 44.94% (40/89), 47.83% (33/69), 56.76% (21/37), and 81.82% (9/11), respectively (Table 2).

The value of FIT or multigene mutation test alone and combination of both tests for CRC screening

A total of 82 CRC, 76 AA, 24 NAA, and 85 healthy subjects were included to verify the sensitivity and specificity of the FIT/multigene mutation test alone and the combination of both tests. The results showed that 59 CRC patients (71.95%) were positively detected by FIT, and 39 patients (47.56%) were positively tested by the multigene mutation test. The positivity rate of the combined test in CRC patients was 84.15%. Among the AA patients, 22 cases (28.95%) were positively tested by FIT, and 20 cases (26.32%) were positively tested by the multigene mutation test. The positivity rate of the combination of the two tests in patients with AA was 48.68%. In NAA and healthy controls, 14 cases were detected positive by FIT, while no positive case was detected by mutation detection kit. The specificity of the fecal immunochemistry and multigene mutation test in NAA and healthy controls was 87.16%. The detailed data are shown in Tables 3 - 5 and Figure 1.

Comparison of the diagnostic performance of the combination of FIT and fecal multigene mutation test with that of serum tumor markers

In this section, 108 subjects were selected, including 67 CRC patients and 41 AA patients. Combined use of FIT and fecal multigene mutation test had a sensitivity of 85.07% for the detection of CRC. The sensitivity of serum CEA, CA125, CA199, and CA724 were 29.85%, 2.99%, 14.93%, and 16.42%, respectively. Combined use of FIT and fecal multigene mutation detection had a sensitivity of 46.34% (19/41) for detection of AA, which was significantly higher than that of the four serum tumor markers (Table 6).

The consistency of mutations in tissue samples and fecal samples

In this part, 122 paired tissue and stool samples were tested at the same time. The results are shown in Table 7. The positive coincidence rate between fecal samples and tissue samples was 90.57% (48/53). The negative coincidence rate was 98.55% (68/69), and the overall

Table1. Mutation sites for the fecal multigene (KRAS-APC-p53-BRAF) mutation test.

Gene name	KRAS	APC	p53	BRAF
Mutation sites	G13D	T1556Nfs*3	R273C	V600E
	G12D	E1309Dfs*4	R282W	V600E2
	G12A	R1450*		V600K
	G12V	R876*		V600D1
	G12S	Q1378*		V600D2
	G12R			V600R
	G12C			
	Q61H			
	A146T			
	A146V			
	A146P			
	A117T			
	A117C			

Table 2. The overall positivity rate of the FIT or multigene mutation test alone and the combination of both tests.

		n	FIT positive n (%)	Multigene positive n (%)	FIT + multigene positive n (%)
All		279	104 (37.28)	59 (21.15)	129 (46.24)
Gender	male	157	63 (40.13)	35 (22.29)	77 (49.04)
	female	122	41 (33.61)	24 (19.67)	52 (42.62)
Age (years)	< 40	36	8 (22.22)	7 (19.44)	11 (30.56)
	40 - 49	37	13 (35.14)	7 (18.92)	15 (40.54)
	50 - 59	89	29 (32.58)	21 (23.60)	40 (44.94)
	60 - 69	69	27 (39.13)	15 (21.74)	33 (47.83)
	70 - 79	37	19 (51.35)	6 (16.22)	21 (56.76)
	> 79	11	8 (72.73)	3 (27.27)	9 (81.82)

coincidence rate was 95.08% (116/122). The tissue test results of 5 samples were positive, and the paired stool test results were negative. The positive sites of these five samples were G12S, G12S, G13D, V600E, and G12S, respectively. Additionally, one fecal sample was G12D positive, and the corresponding tissue sample was negative.

DISCUSSION

The study was mainly to evaluate the accuracy of the fecal multigene mutation (KRAS-APC-p53-BRAF) test for CRC screening. The DNA composition in feces is complex, mainly from intestinal flora, food, and exfoliated intestinal mucosal cells. In this study, we used the streptavidin magnetic beads system to capture purified target sequences and enrich the DNA of intestinal exfoliated cells. The fecal multigene mutation (KRAS-APC-p53-BRAF) test was performed on stool samples from different groups to explore the possibility of use of fecal multigene mutation test for screening of early CRC. The positive detection rate of the fecal multigene mutation test was 47.56% in CRC patients and 26.32% in AA patients. No false-positive samples were detected in NAA patients. The results indicated high specificity of the fecal multigene mutation test. The positive samples are more likely to be CRC and AA, which could be the indication for further enteroscopy with biopsy and open colorectal surgery. However, the use of fecal multigene mutation test alone may lead to missed diagnosis in some patients.

We further comprehensively analyzed FIT and the fecal multigene mutation test results and evaluated whether

Table 3. Positive coincidence rate of FIT/multigene mutation test alone and the combination of both tests in CRC (n = 82).

		n	[a] FIT positive n (%)	[b] Multigene positive n (%)	[c] FIT + multigene positive n (%)	p-value [a] vs. [b] [a] vs. [c]
All		82	59 (71.95)	39 (47.56)	69 (84.15)	< 0.01 < 0.01
Gender	male	45	35 (77.78)	23 (51.11)	41 (91.11)	
	female	37	24 (64.86)	16 (43.24)	28 (75.68)	
Age (years)	< 40	8	6 (75.00)	6 (75.00)	8 (100.00)	
	40 - 49	11	6 (54.55)	5 (45.45)	7 (63.64)	
	50 - 59	20	14 (70.00)	13 (65.00)	19 (95.00)	
	60 - 69	24	17 (70.83)	10 (41.67)	19 (79.17)	
	70 - 79	13	10 (76.92)	3 (23.08)	10 (76.92)	
	> 79	6	6 (100.00)	2 (33.33)	6 (100.00)	
Stage	I	25	16 (64.00)	12 (48.00)	20 (80.00)	
	II	22	18 (81.82)	10 (45.45)	19 (86.36)	
	III	17	13 (76.47)	11 (64.71)	16 (94.12)	
	IV	11	8 (72.73)	3 (27.27)	9 (81.82)	
	unknown	7	4 (57.14)	3 (42.86)	5 (71.43)	

Table 4. The positive coincidence rate for Fit/multigene mutation test alone and the combination of both tests in AA (n = 76).

		n	[a] FIT positive n (%)	[b] Multigene positive n (%)	[c] FIT + Multigene positive n (%)	p-value [a] vs. [b] [a] vs. [c]
All		76	22 (28.95)	20 (26.32)	37 (48.68)	< 0.01 < 0.01
Gender	male	44	15 (34.09)	12 (27.27)	23 (52.27)	
	female	32	7 (21.88)	8 (25.00)	14 (43.75)	
Age (years)	< 40	7	0 (0.00)	1 (14.29)	1 (14.29)	
	40 - 49	7	3 (42.86)	2 (28.57)	4 (57.14)	
	50 - 59	25	11 (44.00)	8 (32.00)	17 (68.00)	
	60 - 69	25	4 (16.00)	5 (20.00)	8 (32.00)	
	70 - 79	10	4 (40.00)	3 (30.00)	6 (60.00)	
	> 79	2	0 (0.00)	1 (50.00)	1 (50.00)	
Tumor size (cm)	< 1	2	0 (0.00)	1 (50.00)	1 (50.00)	
	1 - 2	31	5 (16.13)	5 (16.13)	8 (25.81)	
	2 - 3	26	11 (42.31)	6 (23.08)	17 (65.38)	
	≥ 3	17	6 (35.29)	8 (47.06)	11 (64.71)	

combined use of the two tests can improve the accuracy of existing detection techniques. The results showed that the positivity rate of the combined detection was 84.15% in CRC, which was 12.20% higher than FIT alone. In AA, the positive detection rate with combined use of the two modalities was 48.68%, which was 19.73% higher than that of FIT alone. The specificity achieved with the combined use of both methods was consistent with that of FIT alone (87.16%). The results showed that the combined use of the fecal multigene mutation test and FIT may help improve the sensitivity. In addition, the specificity was 87.16%, which could better predict the risk of CRC and precancerous lesions. Therefore, the combined use of fecal multigene muta-

Table 5. The negative coincidence rate for FIT/multigene mutation test alone and the combination of both tests in NAA and healthy controls (n = 109).

	n	FIT negative n (%)	Multigene negative n (%)	FIT + multigene negative n (%)
All	109	95 (87.16)	109 (100.00)	95 (87.16)
NAA	24	21 (87.50)	24 (100.00)	21 (87.50)
Healthy controls	85	74 (87.06)	85 (100.00)	74 (87.06)

Table 6. Comparison of positivity rates between FIT and fecal multigene mutation combined detection with serum tumor markers.

		FIT + multigene	CEA	CA125	CA199	CA724
	n	positive n (%)	positive n (%)	positive n (%)	positive n (%)	positive n (%)
All	108	73 (67.59)	22 (20.37)	3 (2.78)	11 (10.19)	14 (12.96)
CRC	67	57 (85.07)	20 (29.85)	2 (2.99)	10 (14.93)	11 (16.42)
AA	41	19 (46.34)	2 (4.88)	1 (2.44)	1 (2.44)	3 (7.32)

Table 7. Comparison of detection results between paired tissue samples and stool samples.

Stool	Tissue		All
	positive	negative	
Positive	48	68	116
Negative	5	1	6
All	53	69	122

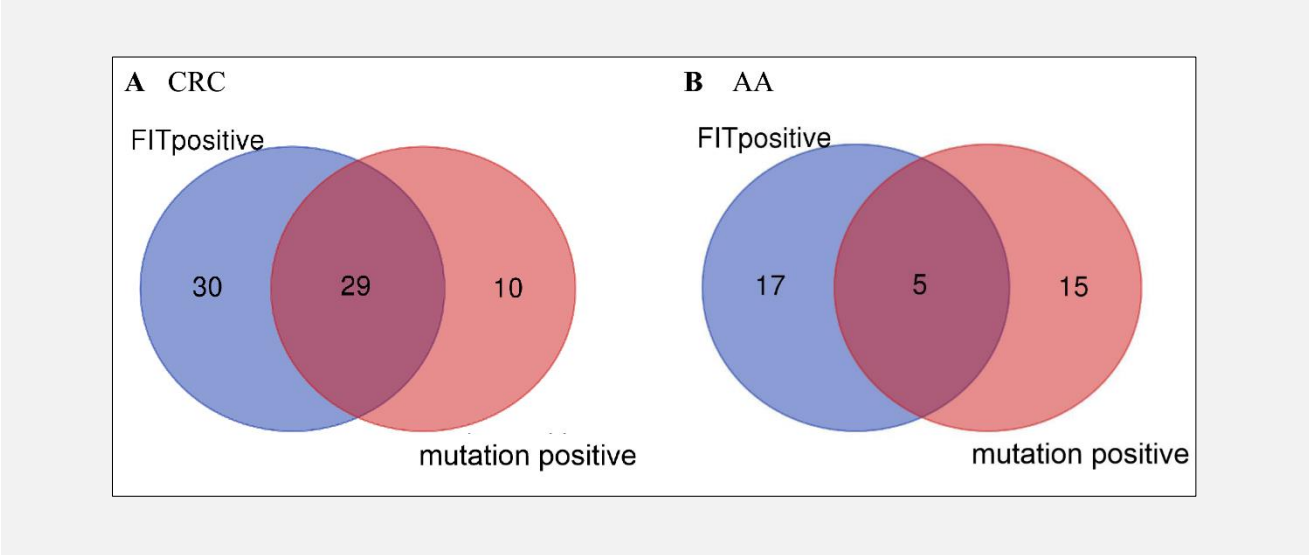

Figure 1. Number of positive samples of FIT/multigene mutation combined tests in colorectal cancer (CRC) and advanced adenoma (AA).

tion test and FIT may offer a distinct advantage for CRC screening. We also compared the sensitivity of the combined use of the fecal multigene mutation and FIT with that of the currently used serum tumor markers, including CEA, CA125, CA199, and CA724 [21-23]. The sensitivity of combined use of FIT and the fecal multigene mutation detection was significantly higher than that of the four serum tumor markers.

In addition, fecal samples and paired tissue samples were detected by fecal multigene mutation test to explore the consistency between fecal and tissue samples and to determine whether fecal samples can be used in place of tissue samples for detection. One hundred twenty-two paired samples were collected for detection. Compared with tissue samples, the positive coincidence rate of fecal samples was 90.57%, and the negative coincidence rate was 98.55%. The detection coincidence rate was higher than that reported in relevant research [24]. At the same time, the detection coincidence rate of the fecal multigene mutation test is also higher than that of plasma and tissue reported in previous studies [25]. Compared with plasma samples, fecal samples showed a higher sensitivity and may potentially replace tissue as an indicator of medication guidance.

In this study, multigene mutation test results of fecal samples were consistent with those of tissue samples. This implies that fecal samples can be used in place of tissue samples for guiding individualized medication for patients. Secondly, the combined use of the fecal multigene mutation test and FIT has a high specificity for detection of NAA. Patients with positive results are more likely to be CRC or AA. Finally, combined use of the fecal multigene mutation test and FIT can help detect more cases of colorectal cancer. The combination of the two tests has higher sensitivity, especially for precancerous and cancerous lesions.

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Declaration of Interest:

We declare that we have no conflicts of interest.

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