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A New Panel of DNA Methylation Biomarkers for Screening Colorectal Cancer and Precancerous Lesions in a Chinese Population

Huang Z[1], Zhao P[2], He Y[3], Sun W[2], Wang Z[1], Dai C[1], Wang Z[1], Wang Z[1], Jin J[1], Zhang T[1] and Ma X[1*]

[1]Department of Oncology, Qingdao Central Hospital, The Second Affiliated Hospital of Medical College of Qingdao University, Qingdao, Shandong, China

[2]Biotherapy Center, Qingdao Central Hospital, The Second Affiliated Hospital of Qingdao University Medical College, Qingdao, Shandong, China

[3]Department of Gastroenterology, Qingdao Central Hospital, The Second Affiliated Hospital of Medical College of Qingdao University, Qingdao, Shandong, China

[*]Corresponding author:

Xuezhen Ma,
Department of Oncology, Qingdao Central Hospital, The Second Affiliated Hospital of Medical College of Qingdao University, No. 127 Siliunan Road, Qingdao, Shandong, 266042, People's Republic of China, Tel: +86-18660229289,
Fax: (86)-532-85953085,
E-mail: 18660229289@126.com

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Abbreviations:

CRC: colorectal cancer; AD: adenomas; HP: hyperplastic polyps; FOBT: fecal occult blood testing; SDC2: syndecan-2; PPP2R5C: protein phosphatase 2, regulatory Subunit B', gamma; ADHFE1: Alcohol Dehydrogenase Iron Containing 1; ROC: Receiver operating characteristic; AUC: area under the curve; CI: confidence interval; qRT-PCR: quantitative real-time Polymerase Chain Reaction

1. Abstract

1.1. Aims The identification of tumor-specific DNA methylation patterns in epithelial colorectal cells in feces has emerged as a novel and noninvasive screening method for colorectal cancer (CRC) and precancerous lesions. Our study investigates a new panel of DNA methylation biomarkers with high sensitivity and specificity for the early screening of CRC and advanced adenomas in a Chinese population.

1.2. Methods Target DNA biomarkers were isolated and subjected to real-time flurescence quantification analysis in fecal samples from 64 CRC patients, 72 advanced adenomas(AA) patients, 33 hyperplastic polyps(HP) patients and 59 healthy controls. Receiver operating characteristic (ROC) curve was performed to compare the diagnosis value of the DNA methylation biomarker panel with traditional fecal occult blood testing(FOBT).

1.3. Results The classification model built with methylation of SDC2, PPP2R5C and ADHFE1 showed a sensitivity of 90.63% for CRC, 59.72% for AA and 21.21% for HP, with the specificity of 91.53%. Fecal DNA methylation test is superior to FOBT for the diagnosis of CRC and AA (AUC: 0.85 vs. 0.71, $P<0.001$), especially for AA (AUC: 0.82 vs. 0.64, $P<0.001$). No noticeable correlation was found between the sensitivity of DNA methylation testing panel and the clinical characteristics. This panel was more accurate than FOBT in detecting CRC and AA for patients >50 years (47.15% vs 73.17%, $P<0.05$).

1.4. Conclusion Our findings validated the methylation testing of SDC2, PPP2R5C and ADHFE1 in feces as a promising alternative for screening colorectal cancer and advanced adenomas.

2. Introduction

Colorectal cancer(CRC) is the third most common cancer and has ranked second in terms of mortality worldwide in 2020 [1]. The five-year survival rate exceeds 90% for patients with lesions still confined to the intestinal wall, but is only 68% for patients with local lymph node involvement and less than 10% if distant metastases occur [2]. Reducing the exposure to risk factors, such as sedentary lifestyle, obesity, smoking and alcohol consumption and unhealthy diatary habits, attributes to decrease the incidence and mortality rates of CRC, but most effectively, we need to improve our screening methods to distinguish those potential patients at early stage, and by polypectomy, to prolong their lifespan [3-5].

Currently, there are mainly two categories of CRC screening for the average-risk population: structual and stool-based examinations. Structual examinations mainly include colonoscopy, double-contrast barium enema (DCBE) and flexible sigmoidoscopy (FSIG); stool tests mainly include occult blood or exfoliated DNA testing [4]. Among them, colonoscopy is the most accurate, it allows visualization of full colon and rectum and can remove the lesions in a single session, which enable it of both diagnosis and treatment value. But colonoscopy also has limititions. It requires several days of unpleasant bowel preparation, and due to its invasiveness and the risk of bowel perfortion, patients are less likely to choose colonoscopy as their screeening option [6]. DCBE can also detects and evaluates entire bowel, and it provides a second chance for patients with contraindications or who have failed colonoscopies. But the extensive bowel preparation, lower sensitivity for adenomas and lack of access to polypectomy or biopsy limit the application within general public. Though FOBT is noninvasive, its sensiticity are ralatively low and different according to the location of the lesions, which may require repeated screening or colonoscopy for further diagnosis [7]. These years, stool-based DNA methylation appears as a emerging approach to detect CRC. As the onset of CRC is the consequence of the accumulation of gene mutations, aberrant DNA methylation and chromatin modifications, which transform normal epithelial cells into colon cancer cells [8], and these neoplastic cells continue to disperse into the colon and mix with feces, it is rational in theory to screen CRC by detecting exfoliated DNA methylation. Besides, this approach requires no diet or bowel preparation. A clear limitition of sDNA test is that its sensitivity depend on the panal of markers which may only identify the majority but not all of CRC. Thus, finding and developing the appropriate panal is the key to accelerate the clinical application.

The DNA methylation biomarkers SDC2 PPP2R5C and ADHFE1 have been reported to be alternative moleculars in cancer screening [9-14]. There is no previous research using these four biomarkers as a panal to detect CRC or AA. In this research, we compare this new panel of DNA methylation biomarkers with FOBT to detect its diagnostic value in CRC and precancerous lesions screening.

3. Materials and Methods

3.1. Study Design

This research was approved by the ethics committee of Qingdao Central Hospital and affiliated Hospital of Qingdao University. A total of 228 participants were recuited from August 1, 2020 to March 1, 2021 in this study, and written consent was obtained. Participants would be excluded when they were found (1) the previous history of CRC or any other cancer;or (2) ungerwent bowel operation, chemotherapy or radiotherapy. Based on the pathology results, paticipants were gathered into four groups: Control group(n=59), HP (size<10mm) group(n=33), AA(size>10mm) group (n=72), and CRC group(n=64). Clinical features including age, sex, tumor location, TNM stage, differentiation degree, histology and pathology results were recorded.

3.2. Stool collection, DNA extraction, bisulfite treatment and qRT-PCR

Stool samples (about 5g) were collected in 50-Ml tubes with 15mL preservative buffer at least 1 day prior to bowel preparation for colonoscopy, then all samples were stored at -80^{o}C. All stool samples in preservative buffer were thawed and homogenized for 1 min with a shaking device. Each tube was centrifuged at 12,000 g for 15 minutes. The TIANamp stool DNA kit (TIANGEN BIOTECH Co., Ltd.) was used to extract human genomic DNA. Bisulfite conversion was performed to discriminate those methylated DNA with a DNA bisulfite conversion kit (TIANGEN BIOTECH Co., Ltd.). NanoDrop 2000(Thermo Scientific, MA, USA) was used to measure the DNA concentration. After purification and conversion, DNA was kept at -20^{o}C. SLAN-96S(SHANGHAI HONGSHI BIOTECH Co.,LTD) was used to perform qPT-PCR, and three replication were conducted for each sample [15].

3.3. Fecal occult blood testing(FOBT)

FOBT (BeckmanCoulter, Fullerton, CA) were performed to all stool samples immediately once obtained. After 1 day, add a drop of peroxide catalyst to the opposite side of the test card. A blue reaction within one minute is considered positive.

4. Statistical Analysis

Twenty-six sites of these three genes were detected. The methylation result was defined as the methylation index(MI). Based on the different number of methylated sites, the result was defined as positive when MI>5 and as negative when MI<5. The logistic regression was built to differentiate risk factors. Chi-aquare test and Fisher exact test were used as appropriate to evaluate the diagnostic values of stool DNA methylation testing and FOBT. Receiver operation curve(ROC), 95% confidence intervals(CI), sensitivity and specificity were implemented to evaluate the diagnostic accuracy. Statistical analysis was performed using Graphpad Prism 8 and IBM SPSS statistics software version 26.0. P values<0.05 were considered to be statistically significant.

5. Results

5.1. Clinical Characteristics of Subjects

Stool samples were obtained from 228 participants who underwent routine colonoscopies at the Qingdao Central Hospital. The participants (aged 61.45±10.63y, 57.5% male) include 59 healthy controls (aged 52.83±12.38y, 44.10% male), 33 HP patients (aged 56.91±10.21y, 57.60% male), 72 AA patients(aged 61.00±8.61y, 54.20% male) and 64 CRC patients(aged 65.09±10.58y, 62.50% male). The mean age of colonoscopy-negative participants was younger than CRC patients, and the percentage of male was higher than female in the CRC group. Of 228 subjects enrolled, 2, 11, 20 and 25 were at stage I, II, III and IV of CRC, respectively. Meanwhile, 59.72% of advanced adenomas and 78.1% of malignant neoplasms were found in the left colon. The clinical information were presented in Table 1.

Table 1: Clinical information of the participants

Characteristics	Control	HP	AA	CRC
Number	59	33	72	64
Sex(%)				
Male	44.1	57.6	54.2	62.5
Female	55.9	42.4	45.8	37.5
Age(mean±SD), years	52.83±12.38	56.91±10.21	61.00±8.61	65.09±10.64
TNM Stage(%)				
I	/	/	/	3.12
II	/	/	/	17.19
III	/	/	/	31.25
IV	/	/	/	39.06
Unavailable	/	/	/	9.38
Differentiation Degree(%)				
Poor	/	/	/	15.63
Moderate	/	/	/	76.56
Well	/	/	/	7.81
Location(%)				
Left colon	/	42.86	59.72	78.1
Right colon	/	57.14	40.28	21.9
Histology				
Tubular	/	/	98	94
Mucinou	/	/	2	6

hyperplastic polyps; AA, advanced adenomas; CRC, colorectal cancer.

Left colon was defined as the rectum, sigmoid, and descending colon; Right colon was defined as the transverse colon, ascending colon and cecum.

5.2. Clinical performance of DNA methlylation and FOBT in colorectal cancer screening

As shown in Table 2, stool-based DNA methylation testing is more sensitive than FOBT in detecting CRC and precancerous lesions(74.26% vs. 47.06%, P<0.05). To be specific, the sensitivity of DNA methylation was 90.63% for CRC, 59.72% for AA, 21.21% for HP, respectively. For FOBT, the sensitivity was 70.31% for CRC, 26.39% for AA, 6.06% for HP. The specificity of DNA methylation and FOBT was up to 91.53% and 89.83%, respectively. ROC was constructed to evaluate the diagnostic value. As shown in Figure 1, DNA methylation showed better performance than FOBT in CRC and advanced adenomas (AUC:0.85 [95% CI: 0.80-0.91] vs. 0.71 [95% CI: 0.64-0.78], P<0.05; Figure 1D). The AUC of the panel reached 0.91 [95% CI: 0.86-0.97]) in CRC detection (Figure 1A), and 0.82 [95% CI: 0.74-0.89]) in AA detection (Figure 1B), respectively. The diagnostic value in detecting polyps was almost equivalant between DNA methylation and FOBT (AUC:0.59 [95% CI: 0.46-0.72] vs. 0.55 [95% CI: 0.42-0.68], P>0.05; Figure 1C).

Table 2: Sensitivity and specificity of the stool-based DNA methylation test and FOBT

	DNA Methylation		**FOBT**		**P-value[a]**
	Positive	Sensitivity(95%CI)	Positive	Sensitivity(95%CI)	
CRC+AA	101/136	74.26%(65.93%-81.20%)	64/136	47.06%(38.51%-55.77%)	0.001
CRC	58/64	90.63%(80.05%-96.13%)	45/64	70.31%(57.42%-80.75%)	0.003
AA	43/72	59.72%(47.49%-70.91%)	19/72	26.39%(17.01%-38.31%)	0.002
HP	Jul-33	21.21%(9.63%-39.40%)	Feb-33	6.06%(1.06%-21.62%)	0.125
	FOBT		**DNA Methylation**		**P-value[a]**
Control	Negative	Specificity(95%CI)	Negative	Specificity(95%CI)	1
	54/59	91.53%(80.59%-96.84%)	53/59	89.83%(78.50%-95.80%)	

CRC, colorectal cancer; AA, advanced adenomas; HP, hyperplastic polyps; FOBT, fecal occult blood testing; sDNA Methylation, stool DNA methylation testing; CI, confidence interval; [a]P-value of the sDNA Methylation compared with FOBT by paired Chi-square test.

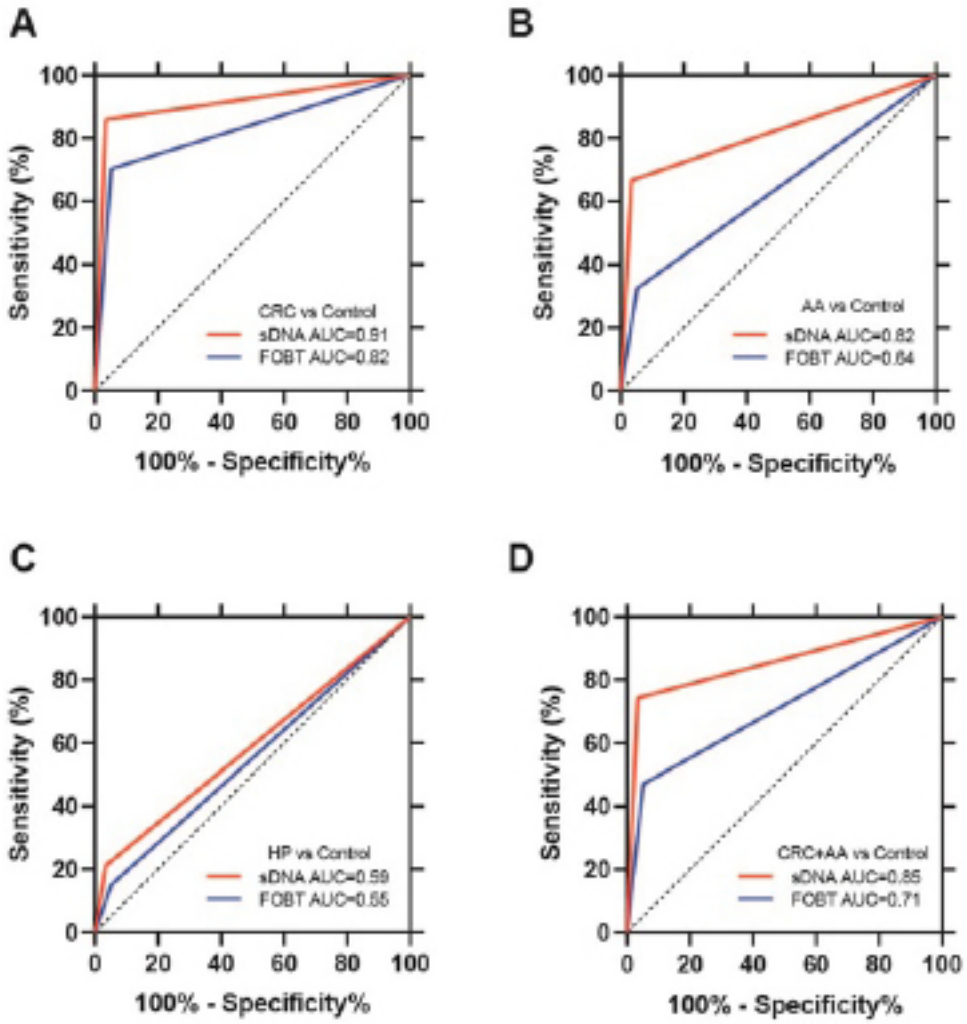

Figure 1

5.3. Associations of clinical-pathologic characteristics with the detection efficiency of the biomarker panel

Based on the logistic regression results, no significant association was found between the detectiong efficiency of the methylation biomarker panel and clinical-pathologic characteristics (age, sex, TNM stage and neoplasm location). In contrast to the methylation test, FOBT results were affected by neoplasm location (Table 3, P<0.05), which was consistent with the findings reported by David et al. [16]. FOBT results showed that the proximal colon is more easily detected than the distal colon (74.00% vs. 57.14%, P<0.05). Age is not an influence factor of the accuracy of multitarget stool DNA test, proving an equal detecting value in the young and the old. Notably, the sensitivity of the DNA detection and FOBT in our study were both superior in screening patients aged between 50 to 60 years (94.44% and 77.78%, respectively). Stratified by age, fecal DNA detection was as accurate for patients ≤ 50y as for those > 50y (76.92% vs. 73.17%) in CRC and AA screening. Compared with FOBT, the detection sensitivity of DNA methylation increased from 46.15% to 76.92% in patients aged ≤50y, although the difference was not statistically significant. Moreover, based on paired Chi-square test, DNA methylation testing is significantly more accurate than FOBT in patients older than 50 years (47.15% vs73.17%, *P*<0.05).

Table 3: Correlation between detection effect and clinical characteristics

Type	Attributions	sDNA methylation				FOBT			
		+	-	Sensitivity	P-value	+	-	Sensitivity	P-value
CRC	**Age(y)**								
	≤50	2	1	66.67%	0.48	2	1	66.67%	0.71
	50-60	17	1	94.44%		14	4	77.78%	
	60-70	19	3	86.36%		14	8	63.64%	
	> 70	19	2	90.48%		14	7	66.67%	
	Sex								

	male	36	4	90.00%	0.66	29	11	72.50%	0.32
	female	22	2	91.67%		16	8	66.67%	
	Differentiation Degree								
	Poor	9	1	90.00%	0.45	7	3	70.00%	0.63
	Moderate	43	6	87.76%		33	16	67.35%	
	Well	4	1	80.00%		3	2	60.00%	
	Location								
	left	45	5	90.00%	0.52	37	13	74.00%	0.016 *
	right	13	1	92.86%		8	6	57.14%	
	TNM stage								
	I	2	0	100.00%	0.13	2	0	100.00%	1
	II	10	1	90.90%		6	5	54.55%	
	III	19	1	95.00%		18	2	90.00%	
	IV	22	3	88.00%		14	11	56.00%	
AA	**Age(y)**								
	≤50	5	5	50.00%	0.49	3	7	30.00%	0.32
	50-60	12	9	57.14%		7	14	33.33%	
	60-70	22	12	64.71%		7	27	20.59%	
	> 70	4	3	57.14%		2	5	28.57%	
	Sex								
	male	23	16	58.97%	0.81	8	31	20.51%	0.33
	Female	20	13	60.61%		11	22	33.33%	
	Location								
	left	26	17	60.47%	0.79	11	32	25.58%	0.74
	right	17	12	58.62%		8	21	27.59%	
CRC+AA	**Age(y)**								
	≤50	10	3	76.92%	0.06	6	7	46.15%	0.98
	> 50	93	30	73.17%		58	65	47.15%	

CRC, colorectal cancer; AA, advanced adenomas; HP, hyperplastic polyps; sDNA Methylation, stool DNA methylation testing; FOBT, fecal occult blood testing; * P < 0.05 by Chi-square test.

6. Discussion

Cancer prevention through screening requires detection of precancerous lesions. Traditionally, adenomas are regarded as the most necessary step to CRC. About more than half of people will develop colorectal adenomas during their lifetime, but merely 6% will undergo malignant transformation [17]. It is the adenomas that are most likely to progress that should be screened for. According to previous studies, those ≥ 1 cm in size or with highly atypical hyperplasia or villous structures are called advanced adenomas; these adenomas have the highest risk of malignant transformation and are often considered as the most relevant part of screening [17,18]. Early screening programs have been proved to be efficient in decreasing the incidence and mortality of colorectal cancer in many countries [19]. In China, FOBT is widely adopted in early detection of CRC, but is restricted due to its low sensitivity and dietary [20]. Previous studies have found fecal DNA methylation testing showed high accuracy of detecting CRC and advanced adenomas [21-23]. Cologuard, which combines an essay for hypermethylated biomarkers(BMP3, NDRG4) and mutant KRAS with FIT test, has been approved by FDA for CRC screening [24]. Multitarget stool DNA test was recmmended by American Cancer Society(ACS) as a new CRC screening option in the updated guideline [25]. Since tumor phenotypes, molecular patterns and genotypes are highly heterogeneous, a single or universal molecular marker for cancer or precancerous lesions is of limited significance and therefore a set of biomarkers are needed.

In this study, we evaluated the diagnostic performances of a new panel of DNA methylation biomarkers, and it showed an overall sensitivity of 90.63% with AUC of 0.91 in detecting CRC not affected by sex, age, location, or tumor stage (P > 0.05), with a specificity of 91.53%. This panel also functioned well in detecting AA (sensitivity 59.72%, AUC=0.82), but had low performance in detecting HP (sensitivity 21.21%, AUC=0.59). We choose three methylation biomarkers (SDC2, PPP2R5C and ADHFE1). Hypermethylated SDC2 was found in all stages of CRC and the stool-based SDC2 methylation test had identical high sensitivity as Cologuard for screening CRC [9]. In a qPCR based mSDC2 assay reported in 2017, the sensitivity of detecting CRC and >1 cm adenomas was 81.1% and 58.2%, respectively, with a specificity of 93.3% [26], which is in line with our findings, implying that SDC2 was a candidate methylation biomarker for CRC screening. Protein phosphatase 2(PP2A) is a serine/threonine phosphatase, which serves as a tumor suppressor by involving in numerous negative signaling pathways of cell growth and division. Inhibition of PP2A activity has been reported to promote malignant transformation [27]. PPP2R5C interacts with PPP2R1A or other PP2A subunits through mutations at the protein–protein binding interface, which may interfere with assembly of the complex and thereby abrogate its cancer suppressive properties [28]. The downregulation and hypermethylation of ADHFE1 in colorectal cancer tissues are associated with poor differentiation and advanced TNM staging in CRC patients [29].

Tumor biomarkers are released into different media at different stages of tumorigenesis. Stool-based DNA testing shows higher sensitivity than plasma-based testing. A meta-analysis revealed that aberrant methylated biomarkers were detected earlier in stool than in blood or urine during CRC progression [30]. This is understandable that DNA in stool samples is directly from the tumor or precancerous lesions while DNA in plasma has to pass through many barriers to finally be release to the blood. Therefore, stool-based testing is more effective and appropriate in screening precancerous lesions and early-stage cancers. In our study, this new panel has better performance than FOBT in screening adenomas and stage I CRC (60.81% vs. 28.38%). Besides, it is reported that stool-based test is often affected by neoplasma sites [30], which is not entirely consistent with our findings. FOBT functioned better in detecting the distal colon than the proximal colon in CRC (74% vs. 57.14%, $P<0.05$), while DNA methylation showed equal efficacy(90% vs. 92.86%, $P>0.05$).

Our study has a few limitations. The number of HP samples, stage I CRC samples was insufficient to evaluate the diagnostic performance, besides, patients with inflammatory bowel disease (ulcerative colitis and Crohn's disease) were also excluded, this could be improved by enrolling more patients in the future studies. Furthermore, this is a retrospective cases with prospective control composite study, multi-center prospective researches should be considered for better and accurate evaluation of fecal DNA metylation test in CRC and its precancerous lesions screening. Further studies are also needed for testing applying intervals of fecal DNA methylation.

In conclusion, our study demonstrated that the new panel of stool-based DNA methylation biomarkers is a potential approach for screening CRC and precancerous lesions, which facilitates the patients who unwillingly undergo colonoscopy and improve their complicance. Further studies are needed to examine the clinical utility of this new panel.

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