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Evaluation of a plasma cell-free DNA methylation test for colorectal cancer diagnosis: a multicenter clinical study

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Abstract

Background A blood-based diagnostic test is a promising strategy for colorectal cancer (CRC). The MethyDT test (IColohunter), which detects methylation levels of *NTMT1* and *MAP3K14-AS1*, exhibited potential in discriminating CRC, but its clinical performance needs to be validated in large-scale populations.

Methods A multicenter, double-blinded, cross-sectional study that enrolled 1194 participants was performed. Plasma samples were collected to detect methylation levels of *NTMT1* and *MAP3K14-AS1* using quantitative methylation-specific PCR with the MethyDT test, and the accuracy was further evaluated by Sanger sequencing.

Results The sensitivities of the MethyDT test for detecting CRC, early stages of CRC (I and II), advanced adenoma (AA), and high-grade intraepithelial neoplasia (HGIN) were 91.2% (95% confidence interval [CI], 88.4–94.0), 87.4% (95% CI, 82.5–92.2), 43.5% (95% CI, 35.7–51.4), and 72.7% (95% CI, 57.5–87.9), respectively. The specificities for participants with non-AA, interfering diseases (ID), and no evidence of disease (NED) were 85.0% (95% CI, 78.8–91.3), 93.7% (95% CI, 91.4–95.9) and 97.3% (95% CI, 90.5–99.7), respectively, and its overall specificity for all-controls was 92.4% (95% CI, 90.3–94.4). The consistency of the MethyDT test with pathology for CRC was high with a kappa value of 0.830 (95% CI, 0.795–0.865). Additionally, the MethyDT test was comparable to Sanger sequencing for detecting methylation with kappa values > 0.97.

Conclusions The MethyDT test demonstrates excellent sensitivity and specificity for CRC and high consistency with Sanger sequencing for methylation, suggesting it may serve as a potential noninvasive diagnostic tool for the detection of CRC.

Trial registration This clinical trial has been registered in ClinicalTrials.gov (NCT05508503).

Keywords Colorectal cancer, Cell-free DNA, Methylation, Liquid biopsy

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Background

Colorectal cancer (CRC) is the third most commonly diagnosed cancer and the second leading cause of cancer-related deaths worldwide [1, 2]. Most CRCs arise from precursor lesions such as adenomatous polyps or sessile serrated lesions [3]. Typically, it takes an estimated 10–15 years for a polyp to progress to CRC, rendering a window of opportunity to detect both early cancer and precursor lesions [4]. Hence, targeted interventions, such as screening and early detection of CRC in high-risk populations are urgently needed to reduce the number of patients with CRC in the coming decades.

Colonoscopy, which can reduce CRC incidence and mortality, is the gold standard for the screening and diagnosis of CRC and adenoma [5–7], but its adherence rate is compromised greatly due to its invasiveness, high cost, patient discomfort, extensive bowel preparation and risk for complications. Non-invasive stool-based screening or diagnosis approaches, such as guaiac-based fecal occult blood tests (FOBT) or hemoglobin-based fecal immunological tests (FIT) are much more cost-effective and convenient than colonoscopy, but their sensitivity for detecting advanced adenoma (AA) is limited [8, 9].

Aberrant DNA methylation contributes to tumorigenesis and progression, occurring early and frequently during CRC development. In recent years, liquid biopsy has become a non-invasive alternative for screening and diagnosing CRC through the use of methylated DNA biomarkers in the circulation [10–15]. To date, circulating tumor DNA (ctDNA) methylation-based biomarkers in the plasma such as *SEPT9*, *APC*, *RASSF2A*, *BCAT1*, and *IKZF1*, have been extensively investigated for clinical application [16, 17]. The *SEPT9* blood test is currently the only plasma test for CRC screening approved by the US Food and Drug Administration based on quantitative methylation-specific PCR (qMSP). However, its sensitivities for detecting CRC, particularly for polyps, were compromised in most studies [14, 18, 19]. Therefore, an accurate blood-based test for early detection of CRC and precancerous lesions remains appealing and imperative, with the benefit of patient convenience, which may improve the adherence.

In a previous study, we found that the methylation levels of both the *NTMT1* gene and *MAP3K14-AS1* gene in CRC samples were significantly higher than those in non-CRC samples [20]. In fact, although the molecular mechanism underlying CRC remains largely unknown, considerable evidence has suggested a close relationship between the two genes and CRC. It has been reported that the methylation level of *MAP3K14-AS1* in CRC samples is significantly higher than that in control samples, indicating its potential as a biomarker for CRC [21, 22]. *NTMT1* has been reported in several studies as a DNA methylation biomarker to distinguish between CRC and non-CRC samples, also demonstrating high diagnostic performance [23–25]. We therefore developed a dual-target methylation test called the MethyDT test (IColohunter) to improve the sensitivity for detection of CRC in plasma samples. However, the clinical performance of the MethyDT test has not been verified in a large population. In this study, we carried out a double-blinded, cross-sectional study at five clinical centers to comprehensively evaluate the clinical performance of the MethyDT test in diagnosing AA and CRC at various stages, and the accuracy of the MethyDT test for methylation detection was compared with Sanger sequencing. In addition, the sensitivity and specificity of the MethyDT test for CRC were further compared with common serum tumor markers such as carbohydrate antigen 19–9 (CA19-9) and carcinoembryonic antigen (CEA) in paired samples.

Methods

Study design

A multicenter, double-blinded, cross-sectional study was performed in five class A tertiary hospitals in China from November 2022 to July 2023. This clinical trial has been registered and released in ClinicalTrials.gov (identifier NCT05508503). The primary objective of the clinical study was to evaluate the performance of the MethyDT test in diagnosing CRC and AA. The secondary objective was to assess the accuracy of methylation detection using the MethyDT test by comparing it with the Sanger sequencing.

Study population

This clinical study recruited patients with CRC, AA or non-advanced adenoma (non-AA), patients with malignancies other than CRC or with benign diseases of the digestive tract (interfering diseases, ID), and individuals with no evidence of disease (NED). Participants with negative colonoscopies and no previous diagnosis of significant disease were considered to have NED. The inclusion criteria of this study were as follows: (1) Patients who required a colonoscopy after initial diagnosis and planned to have an upcoming colonoscopy. All NED participants were enrolled from the participants who met the inclusion criteria (1). (2) Patients who had a diagnosis of primary CRC or AA by colonoscopy and biopsy without any treatment. (3) Patients with untreated digestive system malignancies other than CRC or untreated non-digestive system malignancies. Enrolled participants should meet at least one of the inclusion criteria (1), (2), and (3). The detailed explanation of the inclusion criteria was in Additional File 1: Supplementary Methods. Exclusion criteria were listed below: (1) Cancerous patients received any treatment, such as surgical resection, radiotherapy,

or chemotherapy. (2) Patients with CRC combined with other malignancies. (3) Individuals without a definitive diagnosis. (4) Individuals whose samples were unsuitable for methylation detection or analysis, such as hemolytic samples and invalid samples with ineligible Ct values. Of note, if the Ct values of the internal reference gene *ACTB* in samples are greater than 35, these samples will be excluded due to unqualified (insufficient) cfDNA content. Subjects meeting any of the above exclusion criteria needed to be excluded.

Clinical procedures

For patients with CRC, AA, non-AA, or other diseases, sampling should be done prior to radical resection, radiotherapy, chemotherapy, or other treatments. Tissue biopsies and/or postoperative pathology were reviewed by two pathologists who performed accurate histological classification, tumor staging, and grading. With regard to a participant with two or more colorectal lesions, only the most advanced lesion and its location were considered for categorization. In addition, 85 CRC patients from all five clinical centers were randomly selected for postoperative evaluation with the MethyDT test; blood samples were taken from these patients 4–14 days after surgery. Moreover, serum CEA and CA19-9 results of the participants before treatment were recorded. The staging and grading information of CRC patients and the definition of AA, distal tumors, and proximal tumors were demonstrated in Additional File 1: Supplementary Methods. In particular, gastroenterologists and pathologists were not informed of the MethyDT test result for each participant until unblinding.

The MethyDT test procedures

The MethyDT test (IColohunter) was developed by Wuhan Ammunition Life-Tech Company, China, comprising reagents for plasma cfDNA extraction, bisulfite conversion, and quantitative methylation-specific PCR detection. The detailed detection procedures were described in Additional File 1: Supplementary Methods.

Clinical performance

Using colonoscopy and/or pathology as the reference standard, the clinical performance of the MethyDT test in diagnosing CRC was described by its sensitivity, specificity, and area under the ROC curve (AUC) with 95% confidence intervals (CI). Details were revealed in Additional File 1: Supplementary Methods. Besides, the diagnostic concordance rates between the MethyDT test and clinical diagnosis were calculated and expressed as the kappa value and 95% CI.

Sample size and statistical analysis

The minimum sample size was estimated with a two-sided alpha value of 0.05 and a power of 0.8. The acceptability criteria of sensitivity and specificity referred to the *SEPT9* blood test [26, 27]. While, according to the previous data, the predicted sensitivity of the MethyDT test for CRC and AA, and the predicted specificity for controls were 83%, 40%, and 90%, respectively [20]. The minimum sample size of 212, 97, and 107 were required for CRC patients, AA patients, and controls, respectively. Finally, 398 patients with CRC, 154 patients with AA, and 642 controls were included in the primary analysis. $P < 0.05$ was considered statistically significant. All analyses were performed with SPSS 25.0 (IBM Corporation, USA). Additional data analysis details were in Additional File 1: Supplementary Methods. In addition, all authors had access to the study data and had reviewed and approved the final manuscript.

Results

Characteristics of participants

As shown in Fig. 1 and Table 1, valid results from 1,194 participants who met the screening criteria were utilized to evaluate the clinical performance of the MethyDT test. All participants were classified into 5 categories, which were patients with CRC, AA, non-AA, ID, and subjects with no evidence of diseases (NED), respectively. In addition, Sanger sequencing was performed on 320 plasma samples to assess the accuracy of the MethyDT test in methylation detection. The study included 690 male participants (57.8%) and 504 female participants (42.2%), with a median age of 58 years. Besides, the median ages of CRC, AA, non-AA, ID, and NED participants were 62, 59.5, 57, 53, and 44 years old, respectively. The demographic characteristics of participants at each clinical center were displayed in Additional File 2: Table S1, while the number of participants with different types of ID at each center was listed in Additional File 2: Table S2.

The MethyDT test demonstrates excellent clinical performance for CRC/AA diagnosis

The methylation status of *NTMT1* and *MAP3K14-AS1* in the plasma of all participants was detected by qMSP with the MethyDT test, and the obtained Ct values were displayed and analyzed in Additional File 3: Fig. S1. The plasma methylation levels of *NTMT1* and *MAP3K14-AS1* were found to be significantly higher in participants with CRC or AA compared to those with non-AA, ID, or NED ($P < 0.05$).

Then, the clinical performance of the MethyDT test was analyzed. As shown in Table 2, the MethyDT test achieved sensitivities of 91.2% (95% CI, 88.4–94.0), 87.4%

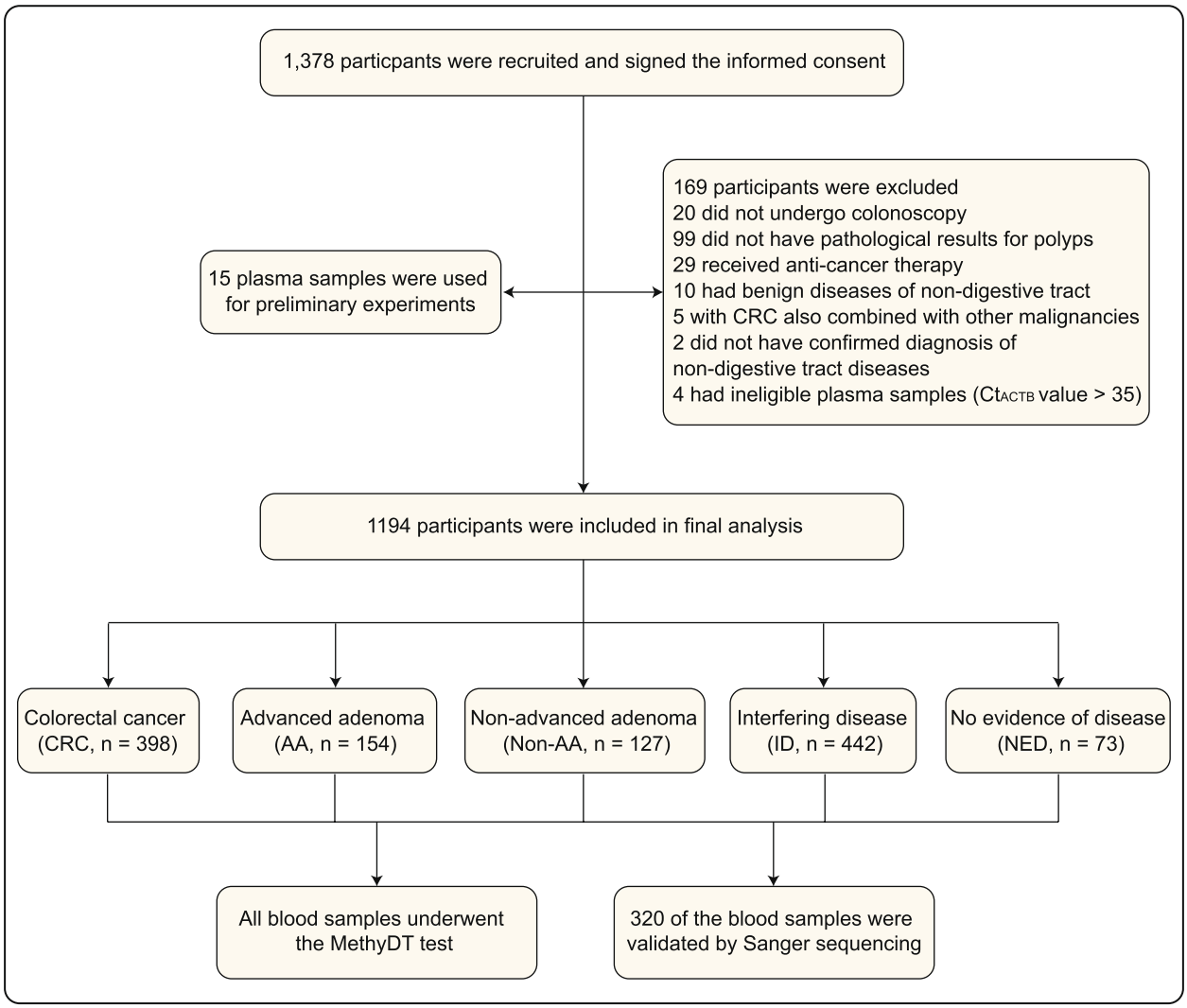

Fig. 1 Flowchart of the clinical study. Of the 154 subjects with advanced adenoma, 33 had HGIN. In addition, 238 participants, including 143 CRC patients, 43 AA patients and 52 controls, had CA19-9 and CEA results before undergoing colonoscopy

(95% CI, 82.5–92.2), and 43.5% (95% CI, 35.7–51.4) for participants with CRC, early stages of CRC (stages I & II) and AA, respectively. In particular, its sensitivity for high-grade intraepithelial neoplasia (HGIN), a category of AA, reached to 72.7% (95% CI, 57.5–87.9). The MethyDT test targeting both *NTMT1* and *MAP3K14-AS1* had higher positive rates for CRC, early stages of CRC, and AA groups than either of these two genes as the target ($P < 0.05$), respectively. Participants who did not have CRC or AA, i.e., those with non-AA, ID, or NED, were assigned to the "all-controls" group. The MethyDT test demonstrated an overall specificity of 92.4% (95% CI, 90.3–94.4). Gradually increasing specificities could be found for non-AA, ID, and NED groups, with specificities of 85.0% (95% CI, 78.8–91.3), 93.7% (95% CI, 91.4–95.9), and 97.3% (95% CI, 90.5–99.7), respectively. Meanwhile, there was no significant difference in specificity between targeting *NTMT1* and *MAP3K14-AS1* and targeting either of these two genes. In addition, the sensitivities and specificities of the MethyDT test for all centers were exhibited in Additional File 2: Table S3 and were comparable to the overall performance shown in Table 2.

Furthermore, the clinical performance of the MethyDT test for different subgroups of CRC, AA, or controls was investigated. The test showed sensitivities ranging from 75 to 100% for detecting various subgroups of CRC (Table 3). Additionally, the sensitivity of the MethyDT test did not significantly vary ($P > 0.05$) according to CRC patients' age, sex, and the grading, histological type, and location of tumors. Nevertheless, it did vary according to

Table 1 Demographic distribution of the study participants

Group	*n*	Sex		Age distribution				
		Male (*n*, %)	Female (*n*, %)	Median (IQR)	≤49 (*n*, %)	50–59 (*n*, %)	60–69 (*n*, %)	≥70 (*n*, %)
All participants	1194	690 (57.8)	504 (42.2)	58 (48, 66)	327 (27.4)	356 (29.8)	332 (27.8)	179 (15.0)
CRC	398	235 (59.0)	163 (41.0)	62 (55, 69)	46 (11.6)	113 (28.4)	141 (35.4)	98 (24.6)
Stage I	73	43 (58.9)	30 (41.1)	62 (56.5, 70)	5 (6.8)	19 (26.0)	28 (38.4)	21 (28.8)
Stage II	109	75 (68.8)	34 (31.2)	62 (56, 69)	9 (8.3)	36 (33.0)	41 (37.6)	23 (21.1)
Stage III	147	83 (56.5)	64 (43.5)	61 (54, 69)	20 (13.6)	40 (27.2)	54 (36.7)	33 (22.4)
Stage IV	25	9 (36.0)	16 (64.0)	60 (54, 68.5)	4 (16.0)	7 (28.0)	9 (36.0)	5 (20.0)
Unknown	44	25 (56.8)	19 (43.2)	62 (52, 74)	8 (18.2)	11 (25.0)	9 (20.5)	16 (36.4)
AA	154	109 (70.8)	45 (29.2)	59.5 (51, 67)	30 (19.5)	47 (30.5)	51 (33.1)	26 (16.9)
Non-AA	127	76 (59.8)	51 (40.2)	57 (51, 63)	23 (18.1)	57 (44.9)	32 (25.2)	15 (11.8)
ID	442	241 (54.5)	201 (45.5)	53 (40, 61)	182 (41.2)	126 (28.5)	99 (22.4)	35 (7.9)
DSMotCRC	80	55 (68.8)	25 (31.3)	63.5 (56, 69)	11 (13.8)	19 (23.8)	34 (42.5)	16 (20.0)
Non-DSM	94	37 (39.4)	57 (60.6)	59.5 (51, 67)	19 (20.2)	28 (29.8)	31 (33.0)	16 (17.0)
BDDT	268	149 (55.6)	119 (44.4)	47 (35, 56)	152 (56.7)	79 (29.5)	34 (12.7)	3 (1.1)
NED	73	29 (39.7)	44 (60.3)	44 (32.5, 56)	46 (63.0)	13 (17.8)	9 (12.3)	5 (6.8)

Colorectal cancer, advanced adenoma, non-advanced adenoma, interfering diseases, and no evidence of disease are abbreviated as CRC, AA, non-AA, ID, and NED, respectively

Digestive system malignancies other than CRC, non-digestive system malignancies, and benign diseases of the digestive tract are abbreviated as DSMotCRC, non-DSM, and BDDT, respectively

Table 2 Diagnostic performance of the MethyDT test

Group	The MethyDT test		*NTMT1*			*MAP3K14-AS1*		
	Positive (*n*)	Sensitivity (95% CI)	Positive (*n*)	Sensitivity (95% CI)	McNemar's test, [a]	Positive (*n*)	Sensitivity (95% CI)	McNemar's test, [b]
CRC	363	91.2 (88.4–94.0)	344	86.4 (83.1–89.8)	$P<0.001$	331	83.2 (79.5–86.9)	$P<0.001$
Early stages of CRC	159	87.4 (82.5–92.2)	149	81.9 (76.3–87.5)	$P=0.002$	132	72.5 (66.0–79.0)	$P<0.001$
AA	67	43.5 (35.7–51.4)	61	39.6 (31.9–47.3)	$P=0.031$	58	37.7 (30.0–45.3)	$P=0.004$
HGIN	24	72.7 (57.5–87.9)	23	69.7 (54.0–85.4)	$P=1.000$	19	57.6 (40.7–74.4)	$P=0.063$
	Negative (n)	Specificity (95% CI)	Negative (*n*)	Specificity (95% CI)	McNemar's test, [a]	Negative (*n*)	Specificity (95% CI)	McNemar's test, [b]
Non-AA	108	85.0 (78.8–91.3)	109	85.8 (79.8–91.9)	$P=1.000$	109	85.8 (79.8–91.9)	$P=1.000$
ID	414	93.7 (91.4–95.9)	418	94.6 (92.5–96.7)	$P=0.125$	416	94.1 (91.9–96.3)	$P=0.500$
NED	71	97.3 (90.5–99.7)	71	97.3 (90.5–99.7)	$P=1.000$	72	98.6 (92.6–100)	$P=1.000$
All-controls	593	92.4 (90.3–94.4)	598	93.2 (91.2–95.1)	$P=0.063$	597	93.0 (91.0–95.0)	$P=0.125$

Early stages of CRC indicate CRC patients at stage I or stage II

High-grade intraepithelial neoplasia is abbreviated as HGIN

All controls comprise participants with non-AA, ID, or NED

[a] Indicates comparisons of the sensitivity or specificity between *NTMT1* and the MethyDT test

[b] Indicates comparisons of the sensitivity or specificity between *MAP3K14-AS1* and the MethyDT test

the TNM stages ($P=0.040$). As shown in Table 4, in terms of patients with AA, the sensitivity of the MethyDT test also did not change obviously according to the subject's sex, age, size of the largest AA, number of AA, and location of AA ($P>0.05$). However, compared to tubular adenoma, the positive detection rates for villus-containing adenoma were much higher (56.1% vs 28.1%, $P=0.001$). Specificities of the MethyDT test for subgroups of ID and for subgroups of all-controls with different sex or age were presented in Additional File 2: Tables S4 and S5, respectively. The range of specificities was between 86.3 and 95.7%.

As displayed in Fig. 2, ROC curves were constructed to analyze the comprehensive performance of each test.

Table 3 Sensitivities of the MethyDT test for CRC patients grouped by sex, age, TNM staging, grading, histological types, and location of tumors

Group	*n*	Positive (*n*)	Sensitivity (95% CI)	Chi-square test, *P* value
Sex				
Male	235	218	92.8 (89.5–96.1)	*P* = 0.187
Female	163	145	89.0 (84.2–93.8)	
Age				
≤ 49	46	41	89.1 (80.1–98.1)	*P* = 0.444
50–59	113	105	92.9 (88.2–97.6)	
60–69	141	125	88.7 (83.4–93.9)	
≥ 70	98	92	93.9 (89.1–98.6)	
TNM staging				
Stage I	73	61	83.6 (75.1–92.1)	*P* = 0.040
Stage II	109	98	89.9 (84.3–95.6)	
Stage III	147	139	94.6 (90.9–98.2)	
Stage IV	25	25	100 (86.3–100)	
Unknown	44	40	90.9 (82.4–99.4)	
Histological types of colorectal adenocarcinoma				
NOS	340	308	90.6 (87.5–93.7)	*P* = 0.055
Other types	50	49	98 (89.4–99.9)	
Unknown	8	6	75 (34.9–96.8)	
Grading of colorectal adenocarcinoma NOS				
Low-grade	231	206	89.2 (85.2–93.2)	*P* = 0.353
High-grade	46	44	95.7 (85.2–99.5)	
Unknown	63	58	92.1 (85.4–98.7)	
Location of tumor				
Distal	65	60	92.3 (85.8–98.8)	*P* = 0.360
Proximal	315	285	90.5 (87.3–93.7)	
Unknown	18	18	100 (81.5–100)	

Not otherwise specified is abbreviated as NOS

When comparing the CRC, early stages of CRC, AA, and HGIN with the all-controls, the MethyDT test achieved AUC values of 0.918 (95% CI, 0.898–0.938), 0.899 (95% CI, 0.868–0.929), 0.679 (95% CI, 0.626–0.732) and 0.825 (95% CI, 0.735–0.916), respectively. The AUC values obtained by the MethyDT test were higher than those of *NTMT1* or *MAP3K14-AS1* as a single target in CRC and early stages of CRC versus all-controls, respectively ($P < 0.05$). Using the NED group as a control, the clinical performance of the MethyDT test was shown in Additional File 2: Table S6.

The consistency between the MethyDT test and pathology for CRC and early stages of CRC detection was excellent (kappa values > 0.75). However, the kappa values for AA and HGIN were lower than 0.45, indicating poor consistency (Additional File 2: Table S7). Moreover, 85 CRC patients underwent the MethyDT test before and after radical resection, of whom 77 were positive preoperatively but all 85 were negative postoperatively. It seemed that the result of the MethyDT test may indicate the presence of a tumor in real time.

The MethyDT test was comparable to sanger sequencing in terms of accuracy of methylation detection

To assess the accuracy of the MethyDT test, methylation-specific PCR products of plasma samples were subjected to Sanger sequencing, which is considered the gold standard for methylation detection. Three samples for *NTMT1* and four samples for *MAP3K14-AS1* displayed different methylation statuses between the two methods. The Kappa values were 0.980 (95% CI, 0.956–1) and 0.973 (95% CI, 0.946–1.00) for *NTMT1* and *MAP3K14-AS1*, respectively, suggesting excellent consistency between the MethyDT test and Sanger sequencing (Additional File 2: Table S8).

The MethyDT test was superior to CEA and/or CA19-9 in detecting CRC and AA

In total, 238 participants in this study conducted serum CA19-9 and CEA tests simultaneously before they underwent colonoscopy. Additional File 2: Table S9 showed that the sensitivities of the MethyDT test for detecting CRC and AA were significantly higher than those of CA19-9, CEA, and the combination of CA19-9 & CEA ($P < 0.001$), respectively. Additionally, the MethyDT test achieved higher AUC values for CRC and AA than CA19-9, CEA, and CA19-9 and CEA tests ($P < 0.001$), respectively (Additional File 3: Fig. S2).

Discussion

The high incidence and mortality of CRC remains an exigent problem to be addressed [28]. Screening or detecting CRC at curable stages with a blood test instead of a stool test may mitigate the issue of low compliance [29]. In this double-blinded, multicenter, cross-sectional study, the MethyDT test achieved an overall sensitivity of 91.2% (95% CI, 88.4–94.0), an overall specificity of 92.4% (95% CI, 90.3–94.4) and an AUC of 0.918 (95% CI, 0.898–0.938) for the detection of CRC by measuring the methylation levels of *NTMT1* and *MAP3K14-AS1* by qMSP. The MethyDT test demonstrated a barely satisfactory positive detection rate (43.5%) for AA. Whereas, the MethyDT test had higher detection rates for adenomas containing villous component (56.1%) and HGIN (72.7%), which are more likely to progress to cancer than tubular adenomas. Unlike villus-containing adenomas that have higher levels of methylation in the CpG islands, tubular adenomas have relatively low levels of methylation in these regions [20, 30], making detection of tubular adenomas based on methylation difficult.

The diagnostic performance of the MethyDT test appears to be superior to that of the *SEPT9* blood test and

Table 4 Sensitivities of the MethyDT test in diagnosing different subgroups of advanced adenoma

Group	*n*	Positive (*n*)	Sensitivity (95% CI)	Chi-square test, *P* value
Sex				
Male	109	49	45.0 (35.6–54.3)	*P* = 0.573
Female	45	18	40.0 (25.7–54.3)	
Age				
≤ 49	30	10	33.3 (16.5–50.2)	*P* = 0.190
50–59	47	23	48.9 (34.7–63.2)	
60–69	51	19	37.3 (24.0–50.5)	
≥ 70	26	15	57.7 (38.7–76.7)	
Size of the largest AA				
1–2 cm	56	14	25.0 (13.7–36.4)	*P* = 1.000
> 2 cm	9	2	22.2 (2.8–60.0)	
Number of AA				
Single	50	19	38 (24.6–51.5)	*P* = 0.933
Multiple	36	14	38.9 (23.0–54.8)	
Location of AA				
Distal	24	14	58.3 (38.6–78.1)	*P* = 0.499
Proximal	89	45	50.6 (40.2–61.0)	
Conventional types of AA				
Tubular	64	18	28.1 (17.1–39.2)	*P* = 0.001
Villus-containing	82	46	56.1 (44.7–67.0)	

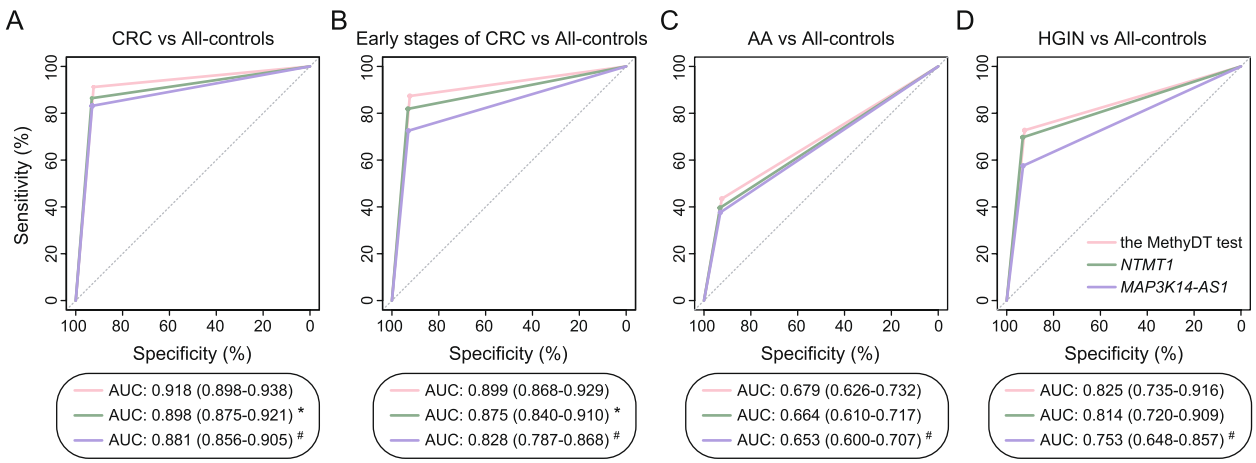

Fig. 2 ROC curves and corresponding AUCs of the MethyDT test, *NTMT1* and *MAP3K14-AS1* for CRC, early stages of CRC, AA, and HGIN vs all-controls, respectively. AUCs between the two groups were compared with the DeLong test. * Indicates a significant difference was found between *NTMT1* and the MethyDT test, # Indicates a significant difference was found between *MAP3K14-AS1* and the MethyDT test. $P < 0.05$ was considered statistically significant

its redesigned version, the Epi proColon 2.0 test [18, 26]. Particularly, the MethyDT test is helpful to find out more patients with AA or CRC in comparison to the Epi proColon 2.0. Furthermore, in a study which enrolled 2105 participants, through detecting the methylation levels of *IKZF1* and *BCAT1* by qMSP, the two-marker blood test acquired a sensitivity of 66% for CRC, a sensitivity of 6% for AA and a specificity of 94% for non-neoplastic pathology cases [17, 31]. Using similar detection methods and algorithms, the MethyDT test showed comparable specificity to the *IKZF1*/*BCAT1*-based blood test, but was more sensitive in detecting CRC and AA. More recently, the assessment of multiple DNA methylation biomarkers in plasma by targeted sequencing or optimized qMSP

has emerged to improve the sensitivity for the detection of CRC and AA. Surprisingly, the performance of the MethyDT test is not inferior to those tests. For instance, it has been reported that a CRC diagnostic model interrogating methylation levels of nine biomarkers achieved a sensitivity of 88% and a specificity of 89% [32]. In cases where the use of only two biomarkers can achieve similar or even better diagnostic performance than using multiple biomarker panels, fewer biomarkers mean that the detection is more convenient and cost-effective. We also compared the performance of the MethyDT test with the MCED test in the CCGA (circulating cell-free genome atlas) work [33]. A binary logistic regression model was constructed using the health status (CRC or non-CRC) of all subjects (except AA) as the dependent variable, and the Ct values (continuous values) of *NTMT1* and *MAP3K14-AS1* as the independent variables. The ROC curve is shown in Additional File 3: Fig. S3. According to this model, when the specificity was set at 99.4%, a level close to the CCGA work (99.3%), we found that the overall diagnostic sensitivity of the MethyDT test for CRC was only 35.2%. Thus, given a similar high specificity, the CCGA work (detection of more than 100,000 informative methylation regions through targeted sequencing) outperforms the MethyDT test in terms of sensitivity for CRC detection. Sequencing and using baits have advantages over qMSP. Meanwhile, we believe that the high specificity threshold used in the CCGA study may result in reduced sensitivity. In our study, slightly lowering the threshold of the specificity (92.4% vs 99.3%) greatly improved the sensitivity (91.2% vs 35.2%) of the MethyDT test for CRC. It may help to detect more precancerous lesions or CRC at an early stage, despite the increased risk of false positive.

We considered that the relatively high diagnostic sensitivity of the MethyDT test for CRC might be attributed to the following reasons. First, the biomarkers we chose had a good ability to distinguish between cancer samples and non-cancer samples. *NTMT1* and *MAP3K14-AS1* were obtained from vast amounts of samples in the public database through the rigorous screening criteria and delicate discover-validation process, and the ability of these two biomarkers to discriminate CRC samples was not inferior to *SEPT9*, *IKZF1*, and *BCAT1*. Additionally, they showed exceptional diagnostic performance in in-house plasma samples [20]. Second, adenoma and early stages of CRC usually release very little ctDNA into the circulation for analysis [34, 35]. In order to maximize the detection of sparsely methylated ctDNA in plasma, suitable qPCR primers and MGB probes were repeatedly screened and validated to improve the amplification efficiency of qMSP [20]. Additionally, we optimized the qMSP reaction system by increasing the quantity of cfDNA input without increasing the collection volume of blood samples and by using a customized 20×PCR buffer. Moreover, we speculated that the double-stranded priming approach (for *NTMT1*) and the dual-MGB probe method (for *MAP3K14-AS1*) improved the sensitivity of the qPCR amplification [20]. Although the short amplicon was also considered an important contributor to improved sensitivity, the lengths of the amplicons in the MethyDT test (101 bp, 64 bp, and 77 bp) were comparable to those of Epi proColon (*SEPT9*, 65 bp) or Colvera (*IKZF1* and *BCAT1*, 95 bp and 102 bp, respectively). Thus, this may not be the reason why the sensitivity of the MethyDT test is superior to the *SEPT9*-, *IKZF1*/*BCAT1*-based assays. Third, the combination of *NTMT1* and *MAP3K14-AS1* as biomarkers and the 1/2 algorithm increased the diagnostic sensitivity compared to the solitary biomarker without decreasing the specificity (Table 2). The choice of controls may have an impact on the specificity of the MethyDT test. For those who are positive for the MethyDT test but negative for subsequent colonoscopy, it may be useful to look for malignant lesions in the digestive system other than the colon. Notably, the MethyDT test had a false-positive rate of only 4.9% in participants with benign diseases of the digestive tract. Accurately differentiating CRC patients from those with benign diseases makes the MethyDT test much more valuable in clinical practice, helping to reduce the misdiagnosis rate and avoid overdiagnosis.

When it comes to CRC screening, we need to take the economic cost–benefit ratio into account. Although it targets two genes, the MethyDT test only requires a single round of triple qPCR rather than three repeated tests, and there are no additional costs for sample collection, processing, DNA extraction, and conversion. We therefore hypothesized that the MethyDT test would not be more costly than the *SEPT9*-based test. Additionally, because of its excellent sensitivity for AA and early stages of CRC, the MethyDT test might be as cost-effective as the *SEPT*9-based test when compared with no screening at all [36, 37]. FIT, which has low sensitivity for adenomas and early stages of CRC, has relatively poor acceptance in the general population due to the requirement for fecal sampling. A blood-based test is expected to yield higher population compliance, which may have an impact on patient benefit. Thus, we look forward to using accurate models to evaluate the cost-effectiveness of the MethyDT test in the near future.

Our study also has several limitations. (1) The age of the controls (participants with NED or benign diseases) was younger than that of CRC patients. We thought that this bias does not affect the clinical performance of the MethyDT test greatly. The median age of patients with non-AA, with digestive system malignancies other than

CRC, or with non-digestive system malignancies was comparable to the median age of the patients with CRC or AA. (2) With only 33 cases of HGIN, the sensitivity of the MethyDT test for diagnosing HGIN needs to be further validated in a large number of participants. (3) Follow-up studies of participants who were the MethyDT test positive but got negative colonoscopy examinations are needed to determine whether they will develop CRC over a certain period of time, which would allow a more comprehensive assessment of the performance of the MethyDT test. (4) Multicenter prospective studies are warranted to evaluate the performance of the MethyDT test in asymptomatic population screening and in opportunistic screening.

Conclusions

The MethyDT test, which detects the methylation levels of *NTMT1* and *MAP3K14-AS1* in plasma samples, demonstrates robust clinical performance for the diagnosis of CRC, early stages of CRC, and AA in a multicenter, double-blinded, and cross-sectional study. Meanwhile, its convenience of sampling and ease of use hold promise for improving the participation rates in the early detection of CRC, so that facilitating the early detection and early treatment of precancerous lesions and CRC.

Abbreviations

CRC	Colorectal cancer
AA	Advanced adenoma
HGIN	High-grade intraepithelial neoplasia
CI	Confidence interval
ID	Interfering diseases
NED	No evidence of disease
ctDNA	Circulating tumor DNA
qMSP	Quantitative methylation-specific PCR
CA19-9	Carbohydrate antigen 19–9
CEA	Carcinoembryonic antigen
AUC	Area under the ROC curve
IRB	Institutional Review Board
FOBT	Guaiac-based fecal occult blood test
FIT	Hemoglobin-based fecal immunological test
MCED	Multi-cancer early detection
CCGA	Circulating Cell-free Genome Atlas

Supplementary Information

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Additional file 1. Supplementary Methods.

Additional file 2: Table S1. Demographic characteristics of participants at each clinical center in this study. Table S2. The number of participants with different types of interfering diseases. Table S3. Sensitivities and specificities of the MethyDT test in participants at each clinical center. Table S4. Specificities of the MethyDT test for participants with interfering diseases. Table S5. Specificities of the MethyDT test for participants with different sex or age. Table S6. Diagnostic performance of the MethyDT test in participants with CRC, early stages of CRC, AA or HGIN in comparison with individuals with NED. Table S7. Consistency of the MethyDT test with clinical diagnosis in detecting CRC or AA. Table S8. Consistency between the MethyDT test and Sanger sequencing in methylation detection. Table S9. Comparison of the MethyDT test, CA19-9, CEA and CA19-9 combined with CEA in diagnosing CRC or AA among 238 participants.

Additional file 3: Fig. S1. Scatter plots of Ct values for *ACTB*, *NTMT1* and *MAP3K14-AS1* in different groups. All valid samples must meet the requirement that the Ct values of *ACTB* ≤ 35, otherwise, the sample was thought to be invalid and no diagnose can be made. The cut-off values for *NTMT1* and *MAP3K14-AS1* were both 48. Data are represented as median with interquartile range (IQR). Columns were compared with Kruskal-Wallis one-way ANOVA test with the Bonferroni correction. $P < 0.05$ was considered statistically significant. Fig. S2. ROC curves and corresponding AUCs of the MethyDT test, CA19-9, CEA, and CA19-9 & CEA tests, for CRC, AA vs all-controls, respectively. AUCs between two groups were compared with the DeLong test. * Indicates a significant difference ($P < 0.001$) was found when compared to the MethyDT test. $P < 0.05$ was considered statistically significant. Fig. S3. ROC curves and corresponding AUCs of the MethyDT test, *NTMT1* and *MAP3K14-AS1* for CRC, early stages of CRC, AA, and HGIN vs all-controls, respectively. The binary logistic regression models were constructed using the health status of all subjects as the dependent variable, and the Ct values (continuous values) of *NTMT1* and *MAP3K14-AS1* as the independent variables. AUCs between two groups were compared with the DeLong test. * Indicates a significant difference was found between *NTMT1* and the MethyDT test, # Indicates a significant difference was found between *MAP3K14-AS1* and the MethyDT test. $P < 0.05$ was considered statistically significant.

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Authors' contributions

ZW, ZH, and XS contributed to original draft writing and formal analysis; DZ, BZ, YL, ZL, and YB contributed to conceptualization and methodology; RL, ZF and XL contributed to investigation, data curation and verification; ZW, ZH, LG, and TX contributed to project administration; YL, ZL, and YB contributed to supervision and writing review. All authors read and approved the final manuscript.

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Availability of data and materials

The data, analytic methods, and study materials are available from the corresponding author upon reasonable request and with the permission of the institution.

Declarations

Ethics approval and consent to participate

All recruited participants signed the informed consent form. The ethics and the clinical study were approved by the Institutional Review Board (IRB) of the five class A tertiary hospitals, respectively. Details could be found in Additional File 1: Supplementary Methods.

Consent for publication

Written informed consent for publication was obtained from the participants or their legally authorized guardians.

Competing interests

DZ, BZ are current employees of Wuhan Ammunition Life-tech Co., Ltd. No potential conflicts of interests were disclosed by the other authors.

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