A Multilocus Blood-Based Assay Targeting Circulating Tumor DNA Methylation Enables Early Detection and Early Relapse Prediction of Colorectal Cancer

Colorectal cancer (CRC) is one of the most common and deadliest cancers worldwide. Patient survival can be greatly improved if cancerous lesions are detected early.[1] Despite a strong recommendation of colonoscopy for CRC screening, patients prefer noninvasive tests, and 83% favor blood-based tests.[2] Blood tests that rely on a single DNA methylation biomarker have been approved[3]; however, they are not recommended due to the low sensitivity for detecting early-stage CRC.[4]

Another unmet clinical need in CRC is the postoperative surveillance for early signs of recurrent disease.[5] Follow-up strategies, such as serum carcinoembryonic antigen (CEA), have limited sensitivity or nonspecific findings.[6,7] More accurate blood-based tests are therefore desired to facilitate monitoring. Here we describe the development, validation, and implementation of ColonAiQ, a multilocus blood test for detecting CRC, advanced adenoma (AA), and CRC early recurrence, as a cost-effective assay for cancer screening and surveillance in blood.

The overall study design is illustrated in Figure 1*A*. To identify DNA methylation markers specific to CRC, we started with a list of 595 cancer-related genomic targets previously curated through a targeted bisulfite sequencing assay, PanSeer (Singlera Genomics (Shanghai) Ltd., Shanghai, China).[8] Methylation profiling of the primary tissues of 60 CRC, 20 AA, and 28 normal tissues revealed the top 150 differentially methylated regions (DMRs), showing a clear separation among these samples (Supplementary Figure 1*A*). These 150 DMRs were then verified in 328 plasma samples and achieved an average area under the curve (AUC) of 0.96 in CRC and 0.86 in the patients with AA, consistently from 100 replications of random sample splitting (Supplementary Figure 1*B*). Based on the insights gained from the PanSeer data, we sought to further narrow down the methylation markers via multiple-step selection procedures to be implemented in a simplified multiplexed quantitative polymerase chain reaction (qPCR) assay easily deployable for clinical use.

After filtering with a set of statistical criteria, 24 markers were selected for evaluation by qPCR in 40 tissue samples (10 CRC, 10 AA, 10 normal, and 10 white blood cells) and 258 plasma samples (142 CRC and 116 healthy). Eventually, 6 markers having higher detection power in tissue and plasma were selected for a multilocus blood test, designated as ColonAiQ (Supplementary Figure 1*C* and *D*).

We then sought to establish a ColonAiQ classifier for early CRC detection based on a case-control study organized from multiple centers (Supplementary Table 1 and Supplementary Figure 1). To cover a full spectrum of clinical conditions, 348 blood samples were collected from 138 patients with CRC, 62 patients with AA, 58 patients with nonneoplastic polyps or other nonneoplastic diseases, and 90 healthy individuals with a negative colonoscopy result. Six markers in ColonAiQ showed a similar performance; thus, a summary score was constructed by averaging the measurements of all makers. This ColonAiQ classifier accurately detected CRC with an average AUC of 0.93 and CRC/AA with an average AUC of 0.84 (Figure 1*B*). The classifier was then locked to validate a second set of 507 plasma samples in a blinded manner. ColonAiQ was able to detect 86% of 173 patients with CRC, with a specificity of 92% in 136 colonoscope-negative controls. In addition, 42% of 107 AAs with a median size of 1.5 cm were successfully identified, whereas the detection for 91 nonadvanced and nonneoplastic polyps remained low (Figure 1*C* and Supplementary Table 1). Increased sensitivity was shown along advancing CRC stages and increasing tumor size (Supplementary Figure 1*E* and *F*).

We next compared the ColonAiQ assay to alternative noninvasive screening methods. Fecal immunochemical testing was previously conducted in 142 tested individuals, including 77 with CRC, 16 with AA, and 49 controls. ColonAiQ outperformed fecal immunochemical testing across all CRC stages (88.3% vs 59.7%), especially for stage I (85.7% vs 28.6%) (Figure 1*D*). Similar superior performance of ColonAiQ was observed in the comparison with CEA, with 80% to 92% and 17% to 47% of early-stage (I/II) CRC samples detected, respectively (Figure 1*E*). When compared with septin 9 (*SEPT9*) the summary score outperformed it in all categories, suggesting that these markers in combination capture a fuller spectrum of tumor epigenetic heterogeneity than individual markers alone (Supplementary Figure 1*G*).

Among all of the patients with CRC, 39 provided blood samples 1 to 2 days before and 17 to 34 days after surgical resection of the tumors. Within 33 patients who were detected positive by ColonAiQ before surgery, 7 (2 stage II and 5 stage III) were confirmed with recurrence that occurred from 42 to 238 days (median, 117 days) after resection. We observed a significant difference ($P = .00017$) in the ColonAiQ score among the postoperative samples between the relapse and nonrelapse groups (Figure 1*F*). Among the recovered patients, 24 of 26 (92%) showed a clear reduction with a tight distribution around the cutoff. In contrast, 6 of the 7 patients with recurrence showed consistently higher scores after the operation, 3 standard deviations away from the mean of the nonrelapse group. Although this was a pilot study on limited cases, our

Abbreviations used in this paper: **AA, advanced adenoma; CEA, serum carcinoembryonic antigen; CRC, colorectal cancer; DMR, differentially methylated region; qPCR, quantitative polymerase chain reaction.**

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A

Marker Discovery

Tissue Screening
- 108 tissues (CRC, AA, normal)
- Processed with ***PanSeer*** assay
- Top 150 markers selected based on CRC/AA against normal

Plasma Validation
- 328 plasmas (from two centers)
- Processed with ***PanSeer*** assay
- The markers confirmed and ranked based on performance in plasma

Marker curations
- Literature reports
- Gene annotation & function
- Location association to genes

Assay development

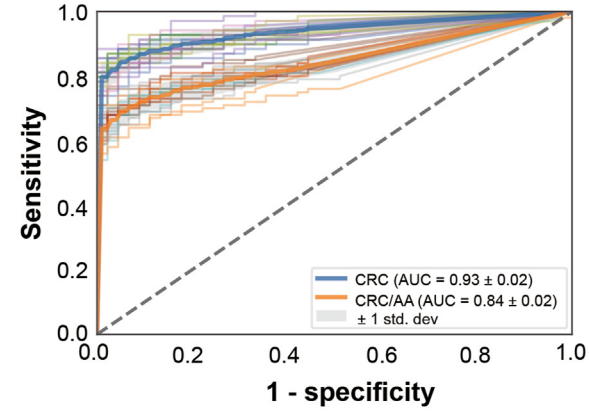
ColonAiQ assay development
- 24 markers selected and processed with qPCR assay
- Verified in 40 tissues and 258 plasma (CRC, AA, normal and/or WBC)
- Markers ranked by their performance to select the top 6 integrated in **ColonAiQ** assay

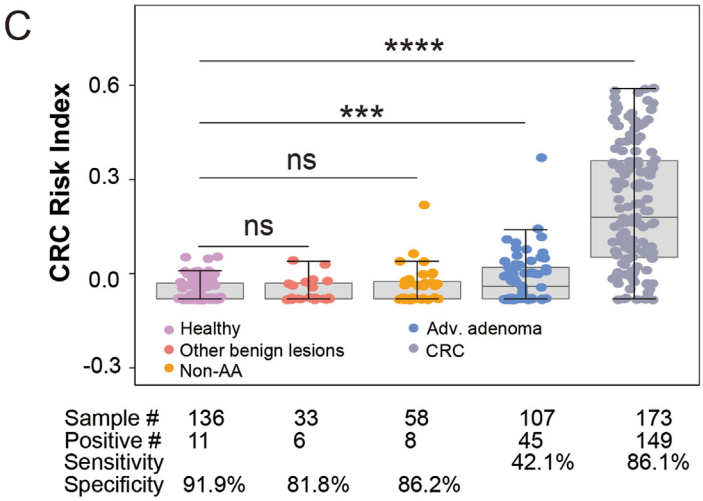
ColonAiQ classifier
- 348 plasma samples (CRC, AA, and controls) selected from multiple centers
- Processed with **ColonAiQ** assay
- Classifier built based on the best performance in CRC and healthy

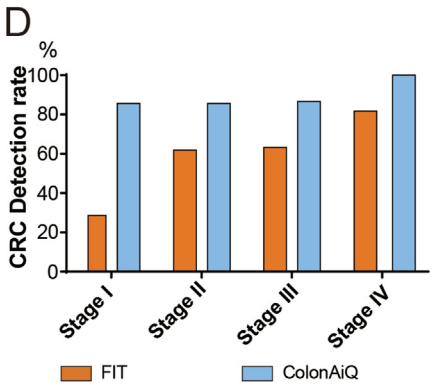
Assay application

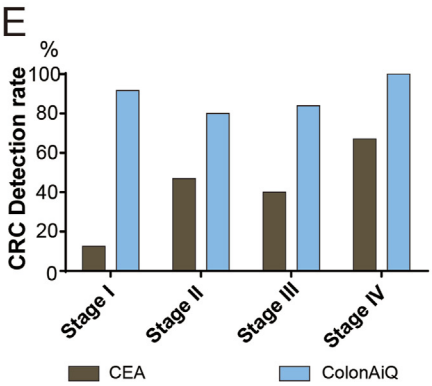
Early detection validation
- 507 Plasma selected from multiple centers
- Processed with **ColonAiQ** assay
- **ColonAiQ** classifier's performance validated in CRC, AA, and controls

Early relapse prediction
- 39 CRC patients were monitored at different time points before and after (17~34 days) the operation
- 7 Patients were confirmed with early relapse by CT image within 8 months; 6 cases showed a high level of ctDNA methylation after operation by **ColonAiQ**









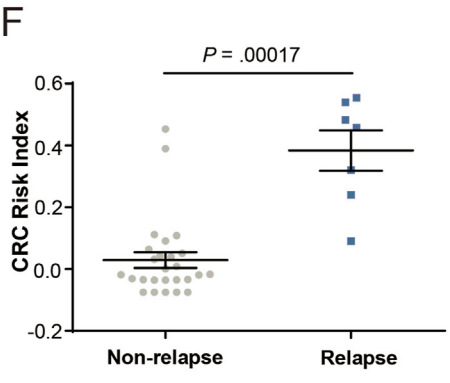


Figure 1. (*A*) An outline of the study design. The study has 3 phases: marker discovery, assay development, and assay implementation. (*B*) Receiver operating characteristic curve of the ColonAiQ assay in the training data set. (*C*) ColonAiQ score distribution of samples in different categories. The sample number, sensitivity, and specificity for each category are presented at the bottom. The Mann-Whitney *U* test was used to compare the methylation level between groups. The *horizontal line* in the middle of each *box* indicates the median; the *top and bottom borders* of the box mark the 75th and 25th percentiles, respectively, the *whiskers* mark minimum and maximum of all the data, and the *circles* outside the whiskers indicate outliers. Not significant (ns): $\geq .05$; ***$P \leq .001$; ****$P \leq .0001$. (*D*) The detection rate for CRC between fecal immunochemical testing (FIT) and ColonAiQ is compared across stages. (*E*) The detection rate for CRC between CEA and ColonAiQ is compared across stages. (*F*) The ColonAiQ score is significantly higher after surgery in patients with early relapse than in those without relapse. The Mann-Whitney *U* test was used to calculate the *P* value.

data suggest that ColonAiQ has the potential to predict early relapse.

To our knowledge, this is the first blood-based qPCR test to integrate 6 CRC methylation markers in 1 assay and outperform the current screening assays. Moreover, the ColonAiQ assay was applied to monitor disease progression after tumor resection in patients with CRC, which represents another potential clinical benefit.

ColonAiQ incorporated several key innovations to overcome technical challenges in early cancer detection. First, multiple iterations of marker screening and validation were conducted to define the most specific methylation targets.

Second, ColonAiQ used the co-methylation patterns of adjacent CpG sites from the same DNA molecule, which improved the signal-to-noise ratio.

Additionally, a combined score allowed robust detection given the presence of tumor epigenetic heterogeneity and molecular dropouts.

Together, these innovations laid the foundation for ColonAiQ's strength. While this study showed the efficacy of ColonAiQ, its performance for screening and prognostic prediction should be confirmed in the future through large-scale prospective trials on an average-risk population and longitudinal follow-up studies for patients with CRC.

In summary, we demonstrated a blood-based circulating tumor DNA methylation assay, ColonAiQ, for the early detection and postoperative surveillance of CRC. As a simple blood-based single reaction workflow, we expect the ColonAiQ assay to be easily implemented in the clinic to reduce the morbidity and mortality of CRC.

Supplementary Material

Note: To access the supplementary material accompanying this article, visit the online version of *Gastroenterology* at www.gastrojournal.org, and at https://doi.org/10.1053/j.gastro.2021.08.054

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Conflicts of interest

These authors disclose the following: Hui Wang, Chengcheng Ma, Zhixi Su, and Rui Liu are employees of Singlera Genomics (Shanghai) Ltd. Rui Liu is a board member of Singlera Genomics and inventor on a patent (US62/657,544) held by Singlera Genomics that covers basic aspects of the library preparation method used in this paper. The remaining authors disclose no conflicts.

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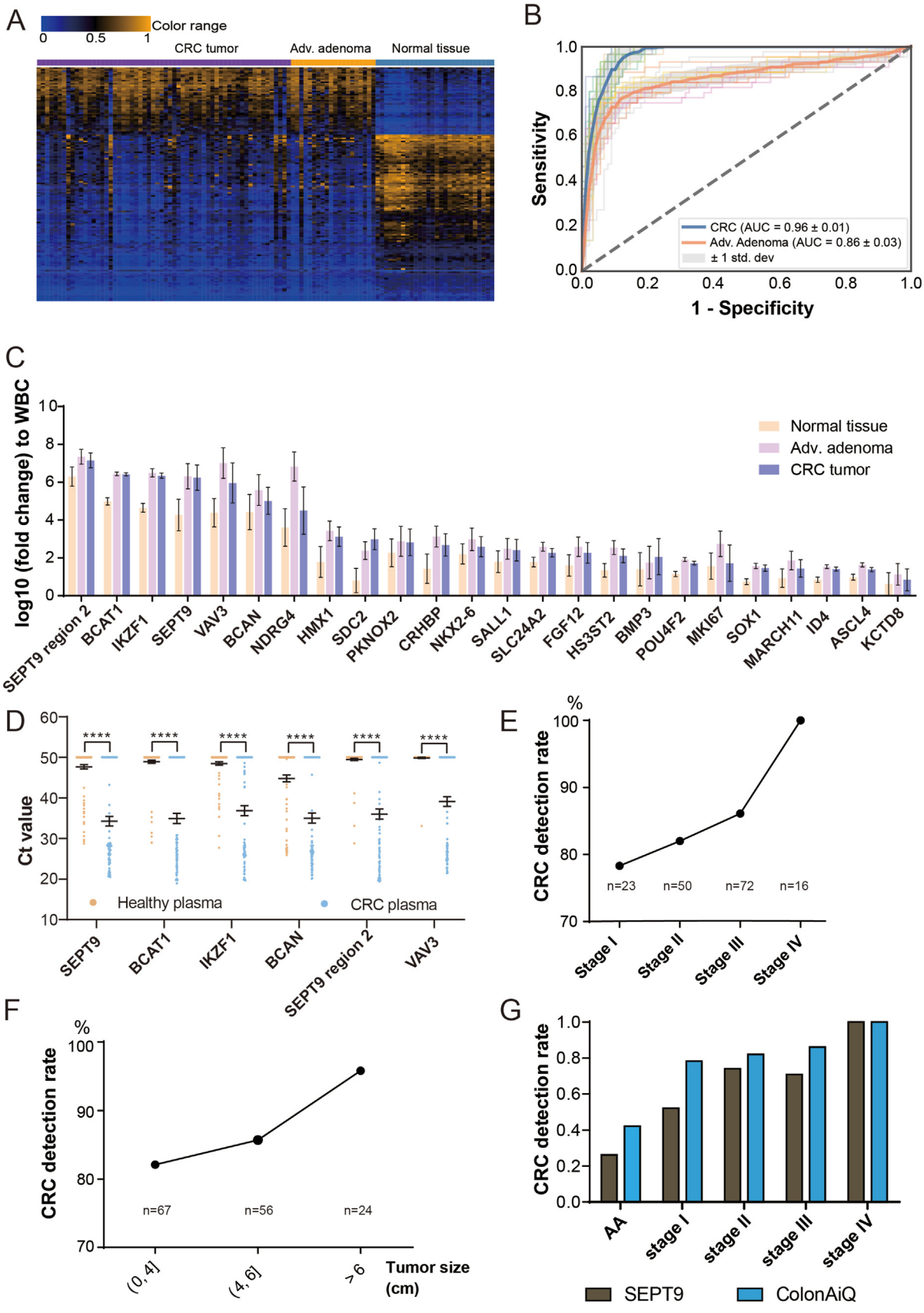
A
Color range
0
0.5
1
CRC tumor
Adv. adenoma
Normal tissue
B
Sensitivity
1 - Specificity
CRC (AUC = 0.96 ± 0.01)
Adv. Adenoma (AUC = 0.86 ± 0.03)
± 1 std. dev
C
log10 (fold change) to WBC
Normal tissue
Adv. adenoma
CRC tumor
SEPT9 region 2
BCAT1
IKZF1
SEPT9
VAV3
BCAN
NDRG4
HMX1
SDC2
PKNOX2
CRHBP
NKX2-6
SALL1
SLC24A2
FGF12
HS3ST2
BMP3
POU4F2
MKI67
SOX1
MARCH11
ID4
ASCL4
KCTD8
D
Ct value
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Healthy plasma
CRC plasma
SEPT9
BCAT1
IKZF1
BCAN
SEPT9 region 2
VAV3
E
%
CRC detection rate
n=23
n=50
n=72
n=16
Stage I
Stage II
Stage III
Stage IV
F
%
CRC detection rate
n=67
n=56
n=24
(0, 4]
(4, 6]
>6
Tumor size (cm)
G
CRC detection rate
AA
stage I
stage II
stage III
stage IV
SEPT9
ColonAiQ

Supplementary Figure 1. (*A*) A heat map shows the methylation pattern of 150 selected markers in the PanSeer tissue data set. (*B*) Receiver operating characteristic (ROC) curve of PanSeer classifier based on plasma data set. The *dark line* shows the average of ROC curve from 100 random sample splits, and the *shaded area* on either side of the ROC curve represents 1 standard deviation. (*C*) The results of markers tested in tissue samples, ranked by $\log_{10}$ (fold change) of CRC to white blood cells (WBC). (*D*) The results (cycle threshold value of qPCR) of the top 6 markers tested in plasma samples. The Mann-Whitney *U* test was used to compare the methylation level of selected markers between groups. ****$P \leq .0001$. (*E*) Influence of stages in CRC detection. (*F*) Influence of tumor size in CRC detection. (*G*) The comparison of the detection rate for CRC between *SEPT9* alone and ColonAiQ across stages.