Efficient detection and post-surgical monitoring of colon cancer with a multi-marker DNA methylation liquid biopsy

Shengnan Jin[a,b,1,2], Dewen Zhu[a,b,1], Fanggui Shao[a,b,1], Shiliang Chen[a,b], Ying Guo[a,b], Kuan Li[a,b], Yourong Wang[a,b], Rongxiu Ding[a,b], Lingjia Gao[a,b], Wen Ma[a,b], Tong Lu[a,b], Dandan Li[a,b], Zhengzheng Zhang[a,b], Suili Cai[a,b], Xue Liang[a,b], Huayu Song[c], Ling Ji[c,d,e], Jinlei Li[c,d,e], Zhihai Zheng[c,d,e], Feizhao Jiang[c,d,e], Xiaoli Wu[f], Ju Luan[a,b], Huxiang Zhang[g], Zhengquan Yang[a,b], Charles R. Cantor[a,h,2], Chang Xu[c,d,e,2], and Chunming Ding[a,b,2]

[a]School of Laboratory Medicine and Life Science, Wenzhou Medical University, 325035 Wenzhou, Zhejiang, China; [b]Key Laboratory of Laboratory Medicine, Ministry of Education, Wenzhou Medical University, 325035 Wenzhou, Zhejiang, China; [c]Department of Colorectal Surgery, The First Affiliated Hospital of Wenzhou Medical University, 325000 Wenzhou, Zhejiang, China; [d]Zhejiang Clinical Research Center of Minimally Invasive Diagnosis and Treatment of Abdominal Diseases, 325000 Zhejiang, China; [e]Wenzhou Clinical Research Center of Colorectal Cancer, Wenzhou Medical University, 325035 Wenzhou, Zhejiang, China; [f]Department of Gastroenterology, The First Affiliated Hospital of Wenzhou Medical University, 325000 Wenzhou, Zhejiang, China; [g]Central Laboratory, The First Affiliated Hospital of Wenzhou Medical University, 325000 Wenzhou, Zhejiang, China; and [h]Department of Biomedical Engineering, Boston University, Boston, MA 02215

Multiplex assays, involving the simultaneous use of multiple circulating tumor DNA (ctDNA) markers, can improve the performance of liquid biopsies so that they are highly predictive of cancer recurrence. We have developed a single-tube methylation-specific quantitative PCR assay (mqMSP) that uses 10 different methylation markers and is capable of quantitative analysis of plasma samples with as little as 0.05% tumor DNA. In a cohort of 179 plasma samples from colorectal cancer (CRC) patients, adenoma patients, and healthy controls, the sensitivity and specificity of the mqMSP assay were 84.9% and 83.3%, respectively. In a head-to-head comparative study, the mqMSP assay also performed better for detecting early-stage (stage I and II) and premalignant polyps than a published SEPT9 assay. In an independent longitudinal cohort of 182 plasma samples (preoperative, postoperative, and follow-up) from 82 CRC patients, the mqMSP assay detected ctDNA in 73 (89.0%) of the preoperative plasma samples. Postoperative detection of ctDNA (within 2 wk of surgery) identified 11 of the 20 recurrence patients and was associated with poorer recurrence-free survival (hazard ratio, 4.20; $P = 0.0005$). With subsequent longitudinal monitoring, 14 patients (70%) had detectable ctDNA before recurrence, with a median lead time of 8.0 mo earlier than seen with radiologic imaging. The mqMSP assay is cost-effective and easily implementable for routine clinical monitoring of CRC recurrence, which can lead to better patient management after surgery.

colorectal cancer | circulating tumor DNA | DNA methylation | recurrence | liquid biopsy

Colorectal cancer (CRC) is one of the most prevalent malignancies worldwide, with morbidity and mortality ranking third and second among all cancers, respectively. Recurrence and metastasis are the main causes of death (1), with ~20 to 25% of patients recurring after surgery (2, 3).

CRC recurrence is monitored by radiologic imaging, colonoscopy, and serum carcinoembryonic antigen (CEA) level (4). CEA analysis is convenient and cost-effective but has low sensitivity (5). Imaging analysis improves the detection of recurrence but may miss small lesions during early recurrence. Patient compliance for colonoscopy is relatively low due to discomfort (6). Additionally, the quality of bowel preparation may impact detection sensitivity.

Circulating tumor DNA (ctDNA) is released by apoptotic tumor cells into body fluids, such as blood. It has emerged as a promising class of minimally invasive biomarkers for early cancer screening, companion diagnosis, and prognosis (7–9). For example,

detection of driver mutations in such genes as *Epidermal Growth Factor Receptor* (*EGFR*) in ctDNA are used to direct tyrosine kinase inhibitor treatment in late-stage non-small cell lung adenocarcinoma (10).

Early detection of recurrence using ctDNA biomarkers is substantially more difficult than using these markers in companion diagnosis. Different cancer patients may carry different somatic mutations. Next-generation sequencing (NGS) can be used to analyze a tumor tissue sample from each patient, and the mutations found can be configured into a custom digital droplet PCR (ddPCR) assay to analyze the patient's ctDNA (11). However, this process is tedious and difficult to standardize. Alternatively, targeted NGS can be performed directly on the ctDNA without prior knowledge of which mutations each patient carries. In this case, a preselected panel of hotspot genes must be sequenced

Significance

Colorectal cancer recurrence is one of the main causes of death. Early prediction of recurrence by minimal residual disease detection and longitudinal tumor monitoring can improve patient outcomes. Next-generation sequencing methods analyzing circulating tumor DNA (ctDNA) in blood can predict recurrence with high accuracy, but these methods are too expensive and complex to be broadly deployable. We have developed a cost-effective and easily implementable single-tube qPCR method (mqMSP) for quantifying a panel of ctDNA methylation markers. Using this panel to monitor patients after surgery can predict cancer recurrence with high accuracy and well in advance of methods in current use.

www.manaraa.com

MEDICAL SCIENCES

at extremely high depth, since somatic mutation allele frequencies may be extremely low (often two orders of magnitude lower than in nontreated late-stage cancer patients) in postoperative or early recurrence plasma samples (12–14).

Abnormal DNA methylation is an early and frequent event in cancer development. Different cancer patients may have different methylation patterns, but there are common DNA methylation changes within each cancer type. For example, *Septin9* gene (*SEPT9*) hypermethylation frequently occurs in CRC and may serve as a universal biomarker for CRC monitoring (15). Indeed, DNA methylation biomarkers have shown potential in tumor screening, prognosis, therapeutic efficacy evaluation, and personalized treatment (16, 17).

DNA methylation biomarkers in ctDNA can be challenging to detect at low levels, since additional processing steps, such as bisulfite conversion, are needed. Single-marker tests, such as *SEPT9* methylation, have low sensitivity for early-stage CRC (18, 19). Combinations of multiple methylation markers (even hundreds to thousands) can increase the sensitivity of cancer detection (20, 21). Others have investigated methylation haplotype blocks for improving tumor detection and tissue-of-origin mapping (22), potentially detecting cancer years before conventional diagnosis (23). In extreme cases, one can analyze the entire methylome of circulating free DNA (cfDNA) for cancer detection (24). In this study, we developed a simple methylation-specific quantitative PCR (mqMSP) assay for ctDNA analysis using 10 subregions of *SEPT9* for the early prediction of CRC recurrence.

Results

Selection and Validation of Multiple DNA Methylation Biomarkers within the *SEPT9* Gene. Single-biomarker analysis with ctDNA suffers from low sensitivity due to the low absolute and relative concentrations of ctDNA in body fluids, particularly in patients with early-stage cancer or early in recurrence. We anticipated that testing multiple DNA methylation markers would significantly improve the detection rate when each single biomarker is present at a concentration close to its detection limit. Additionally, due to individual variations in DNA methylation patterns, a single marker might not be universally applicable to all. Different markers may complement each other to achieve a better overall detection rate.

We chose to interrogate the entire 2-kb CpG-rich region of the *SEPT9* gene promoter, from which a small subregion has previously been used for CRC screening (18, 25), to identify many more potential subregions for ctDNA analysis. After a series of DNA methylation analyses by bisulfite cloning and sequencing, and SYBR Green-based MSP assays using several different types of samples (CRC and paired surrounding normal tissues, buffy coat), we identified 10 subregions to use for further analysis.

TaqMan probe-based qMSP assays were designed for each of the 10 selected subregions (Fig. 1*A*). An internal control assay
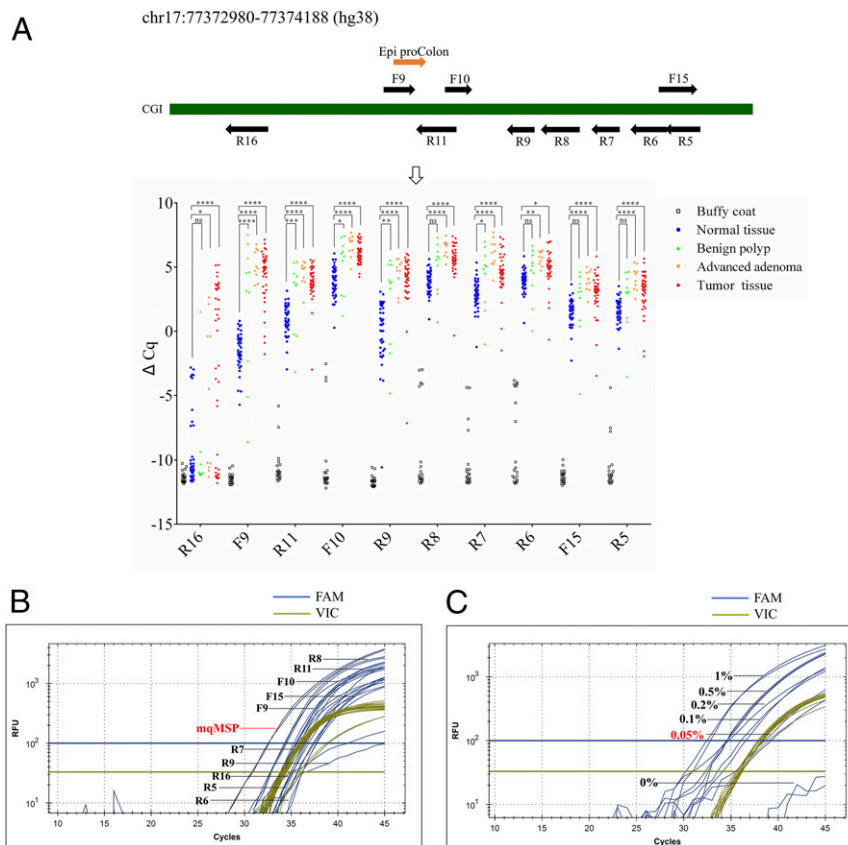

Fig. 1. Selection and validation of multiple DNA methylation biomarkers within the *SEPT9* gene. (*A, Upper*) Chromosomal locations of the 10 selected subregions within the *SEPT9* gene. The 10 subregions are shown with black arrows; forward arrows are for the qMSP assays designed for the forward strand, and reverse arrows are for the assays designed for the reverse strand. The Epi proColon assay is shown in an orange arrow. (*A, Lower*) Samples from 40 pairs of CRCs (red) and surrounding normal tissues (blue), 10 advanced adenomas (orange), 10 benign polyps (green), and 20 buffy coats (black) were analyzed for the 10 selected markers. ****$P < 0.0001$; ***$P < 0.001$; **$P < 0.01$; *$P < 0.05$; ns, not significantly different. (*B*) Comparison between the mqMSP and uniplex qMSP assays. The mqMSP assay produced a ΔCq values that were 3.87, 4.14, 4.62, 4.73, 4.91, 5.90, 6.11, 6.28, 6.77, and 12.54 higher than R8, F10, F9, R11, R7, R16, R5, F15, R6, and R9, respectively. (*C*) Assessment of analytical sensitivity for the mqMSP assay. FAM represents the DNA methylation signal, and VIC represents the internal reference signal. The mqMSP assay detected tumor DNA signals with as little as 0.05% of tumor DNA in the background of buffy coat DNA.

targeting the *ACTB* gene was designed and used in the same reaction with each qMSP assay. The ΔCq value between each qMSP assay and the internal control assay was used to represent the methylation level for each subregion, with a higher ΔCq value representing greater DNA methylation. We selected 40 pairs of CRC and surrounding normal tissues, 10 advanced adenomas, 10 benign polyps, and 20 buffy coat samples for qMSP analyses. For a qMSP assay to be applicable for plasma DNA analysis, the signal strength (represented by the ΔCq value) for buffy coat DNA must be extremely low, since buffy coat-derived cfDNA is the dominant component in total plasma cfDNA. Even weak signals from buffy coat DNA can produce false-positive signals. As shown in Fig. 1*A*, all 10 qMSP assays showed either no or an extremely low background signal in buffy coat DNA, with typical ΔCq values <−10. In the 40 paired CRC and surrounding normal tissue samples, tumor DNA showed significantly higher methylation than paired normal DNA. Advanced adenoma and polyps samples also showed generally higher methylation than normal tissue samples, with more subregions showing a statistically significant difference between advanced adenoma and normal than between polyps and normal, suggesting that the DNA methylation changes may be progressive in tumor development.

mqMSP Assay Development. Our primary aim was to develop a simple qPCR assay for simultaneously quantifying multiple DNA methylation markers for ctDNA analysis from plasma samples. Given the limited probe fluorophore choices, it is not feasible to quantify each subregion individually in a single reaction. Thus, we designed an mqMSP assay with two different fluorophore probes. The FAM fluorophore was used for all 10 subregions, and the VIC fluorophore was used for the *ACTB* control assay. This mqMSP assay measures the total methylation of all 10 subregions.

We compared the 10-marker mqMSP assay with each individual qMSP assay using 10 ng of mixed CRC and buffy coat genomic DNA at a 1:100 ratio. The mqMSP assay produced a

ΔCq value which was 3.87, 4.14, 4.62, 4.73, 4.91, 5.90, 6.11, 6.28, 6.77, and 12.54 higher than the individual R8, F10, F9, R11, R7, R16, R5, F15, R6, and R9 assays, respectively, indicating that the multiplex assay is analytically more sensitive than the individual assays (Fig. 1*B*).

Since ctDNA is often present at low abundance in overall plasma cfDNA, it is critical that the mqMSP assay be able to detect and quantify low proportions of ctDNA in the background of normal DNA. To further evaluate the analytical sensitivity of the mqMSP assay, methylated tumor tissue DNA was mixed with buffy coat DNA at 1%, 0.5%, 0.2%, 0.1%, 0.05%, and 0%, with the total DNA amount fixed at 10 ng per reaction. The DNA mixtures were subject to bisulfite conversion and mqMSP analyses. We observed quantitative detection of tumor DNA at as low as 0.05% of tumor DNA at 10 ng total DNA, mimicking plasma DNA samples from early-stage CRC patients or CRC patients at an early period of recurrence (Fig. 1*C*).

mqMSP Analysis of ctDNA for CRC Detection. To test the performance of the mqMSP assay for detecting CRC using plasma DNA, we recruited 53 CRC patients, 48 patients with advanced adenoma, 30 patients with benign polyps, and 48 healthy controls (as confirmed by colonoscopy). Using the preoperative plasma samples of the 53 CRC patients (Table 1, technical evaluation cohort), we obtained positive mqMSP detection in 84.9% (45/53). Among them, the detection rates for stage I, II, III, and IV patients were 64.3% (9/14), 81.3% (13/16), 100% (9/9), and 100% (14/14), respectively (*SI Appendix,* Table S3). The detection rates for the patients with advanced adenoma, patients with polyps, and healthy controls were 23%, 40%, and 16.7%, respectively (*SI Appendix,* Table S3). The methylation signals of ctDNA were significantly higher in the CRC patients than in the healthy controls. The patients at more advanced stages (III and IV) showed significantly higher methylation signals than those at earlier stages (I and II) (Fig. 2*A*). The area under the curve (AUC) value for the receiver operating characteristic (ROC) curve separating the CRC patients

Table 1.   Clinicopathological characteristics of CRC patients in technical evaluation, comparative study, and longitudinal cohorts

Characteristics	Technical evaluation cohort (n = 53)	Comparative cohort (n = 43)	Longitudinal cohort	
			All patients (n = 82)	Preoperative ctDNA positive (n = 73)
Sex, n				
Male	33	24	53	46
Female	20	19	29	27
Age, y				
Median	69	71	66	67
Range	31 to 90	51 to 89	33 to 85	33 to 85
Localization, n				
Left colon	6	10	8	8
Right colon	3	5	4	4
Rectum	44	28	70	61
Lymph node metastasis, n				
Yes	14	6	36	32
No	34	37	46	41
Unknown	5	0	0	0
Chemoradiotherapy, n				
Yes	38	12	70	61
No	15	31	12	12
Stage, n				
I	14	15	5	4
II	16	17	30	27
III	9	11	40	36
IV	14	0	7	6

Jin et al.
Efficient detection and post-surgical monitoring of colon cancer with a multi-marker DNA methylation liquid biopsy
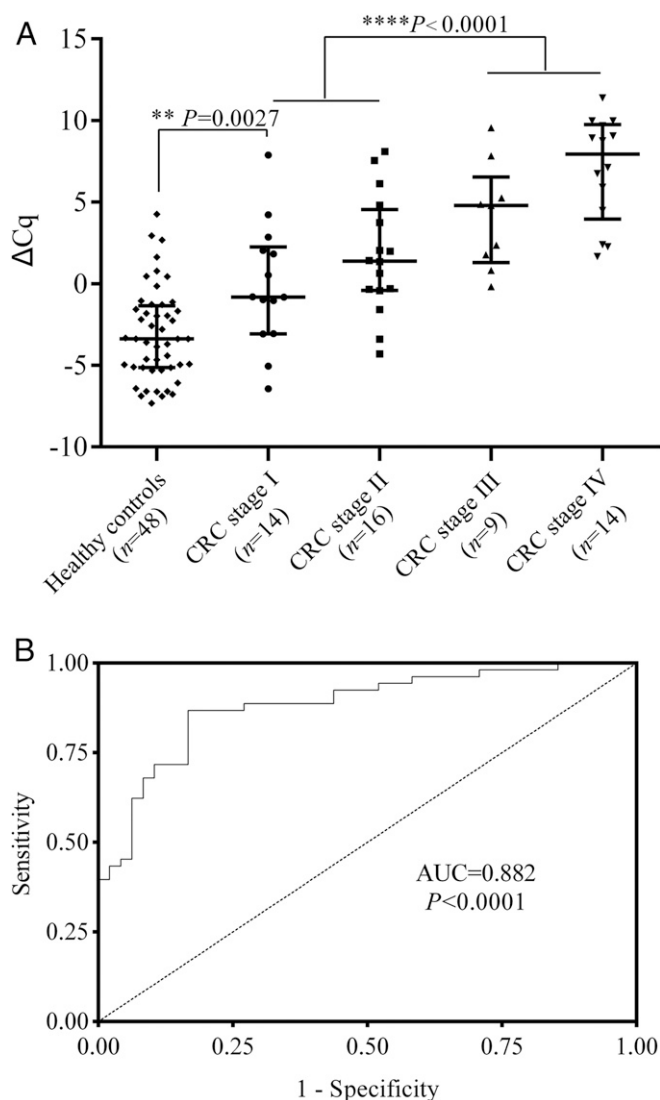
www.manaraa.com

Fig. 2. Quantification of ctDNA by the mqMSP assay in the technical evaluation cohort. (*A*) Methylation levels of plasma DNA as quantified by the mqMSP assay in CRC patients and healthy controls. The *y*-axis represents methylation levels ($\Delta Cq = Cq_{reference} - Cq_{biomarker}$), where a higher value represents a higher methylation level. Lines represent median with interquartile range. The methylation levels were significantly higher in CRC patients than in healthy controls, with significantly higher levels in patients in more advanced stages (III and IV) compared with patients in earlier stages (I and II). (*B*) ROC analysis for separating CRC patients (*n* = 53) and healthy controls (*n* = 48) with an AUC value of 0.882.

and the healthy controls was 0.882 (95% confidence interval [CI], 0.816 to 0.948; *P* < 0.0001) (Fig. 2*B*).

Comparative Analysis of the mqMSP and SEPT9 Assays. The single-marker methylated *SEPT9* gene assay (SEPT9 assay) is now commercially available for blood-based CRC screening. While early studies with relatively small sample sizes reported high sensitivity for CRC detection (15), a large-scale prospective study showed low sensitivity at 11.2%, 35.0%, and 63.0% for advanced adenomas, stage I CRC, and stage II CRC, respectively (18).

We hypothesized that compared with a single-marker approach, our multimarker approach may produce better detection for premalignant and early-stage CRC samples, in which ctDNA may be present at very low levels. To test this hypothesis, we recruited a new cohort of 103 individuals with largely early-stage

CRC, advanced adenoma and benign polyps samples (43 CRC patients, 15 patients with advanced adenoma, 15 patients with benign polyps, and 30 controls with no CRC or polyps). The mqMSP and the SEPT9 assays were performed in parallel for these samples. Overall, the two assays showed highly consistent results (Fig. 3 and *SI Appendix*, Table S4). The mqMSP and SEPT9 assays had the same high specificity at 90%. Compared with the SEPT9 assay, the mqMSP assay had a higher sensitivity for early-stage CRC (73.3% vs. 60% for stage I, 76.5% vs. 70.6% for stage II) and a statistically higher sensitivity for advanced adenoma and polyps (53.3% vs. 26.7% for advanced adenoma, 33.3% vs. 6.7% for polyps; *P* < 0.05, Fisher's exact test). For the benign polyps, advanced adenoma, and early-stage CRC samples, the mqMSP assay was able to detect 13 samples where the SEPT9 assay failed to detect, while the SEPT9 assay detected 2 samples where the mqMSP failed to detect.

mqMSP Analysis of ctDNA in Preoperative and Postoperative Plasma for CRC Recurrence Prediction. To evaluate whether mqMSP analysis can be used for CRC recurrence detection, we prospectively recruited an independent cohort of CRC patients. Eighty-two patients with a minimum of 3 y of follow-up were included for further analysis (Table 1). Among them, 24 patients recurred within 3 y.

Consistent with the results for the first cohort, 73 of the 82 (89.0%) preoperative plasma samples from the CRC patients tested positive in the mqMSP assay. The detection rates were 80% (4/5), 90% (27/30), 90% (36/40), and 85.7% (6/7) for stage I, II, III, and IV patients, respectively. Among them, 20 patients
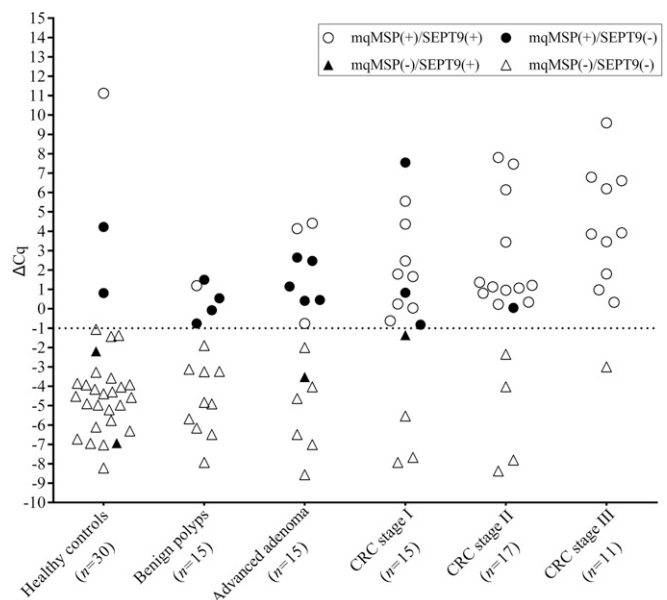

Fig. 3. Comparative analysis of the mqMSP and SEPT9 assays. The mqMSP and SEPT9 assays were performed for the same cohort of 103 individuals (43 CRC patients, 15 patients with advanced adenoma, 15 patients with benign polyps, and 30 controls with no CRC or polyps). A ΔCq value of -1 was used as the cutoff for the mqMSP assay. For the SEPT9 assay, a positive call was made when at least one out of three qPCR replicates had a Ct value <45. Unfilled circles represent samples in which results from both assays were positive, and unfilled triangles represent samples in which results from both assays were negative. The solid circles represent samples in which the mqMSP results were positive and the SEPT9 assay results were negative; the solid triangles represent samples in which the mqMSP results were negative and the SEPT9 assay results were positive. For the benign polyps, advanced adenoma, and early-stage CRC samples, the mqMSP assay was able to detect 13 samples in which the SEPT9 assay failed to detect, while the SEPT9 assay detected 2 samples in which the mqMSP failed to detect.

recurred within 3 y, while 53 patients remained recurrence-free for at least 3 y after surgery. Preoperative ctDNA status or level was not correlated with recurrence.

We next performed mqMSP tests on postoperative plasma samples from the 73 patients with positive preoperative ctDNA. We collected postoperative blood within 2 wk (1 to 14 d) after surgery. Overall, 21 of the 73 patients (28.8%) tested positive in the mqMSP assay of postoperative plasma. Significant decreases in ctDNA levels were observed from preoperative to postoperative plasma, regardless of recurrence status (*SI Appendix*, Fig. S1 *A* and *B*), although nonrecurrence patients had a greater decrease in ctDNA levels measured by $\Delta\Delta Cq$ values [$\Delta\Delta Cq = \Delta Cq(pre\_op) - \Delta Cq(post\_op)$] ($P = 0.009$; *SI Appendix*, Fig. S1*C*).

Among the 20 recurrence patients, 11 (55%) tested positive by the mqMSP assay in the postoperative plasma samples. Positive detection of ctDNA within 2 wk after surgery was associated with poorer recurrence-free survival (RFS; HR, 4.20; 95% CI, 2.30 to 18.73; $P = 0.0005$) (Fig. 4*A*). When patients were stratified by stage, significant RFS differences were observed in localized, stage II, or stage III CRC patients based on postoperative ctDNA status (*SI Appendix*, Fig. S2). Among the 20 recurrence patients, those with positive postoperative ctDNA had a poorer RFS than those with negative postoperative ctDNA (median RFS, 288 d vs. 460 d; $P = 0.008$) (Fig. 4*B*).

Adjuvant chemotherapy is often used for high-risk patients after surgery. Thus, we stratified patients based on adjuvant chemotherapy status. Regardless of the use of adjuvant chemotherapy, positive postoperative ctDNA detection by mqMSP strongly predicted recurrence within 3 y (with adjuvant chemotherapy: HR, 5.16; 95% CI, 2.31 to 29.78; $P = 0.001$; without adjuvant chemotherapy: HR, 4.08; 95% CI, 1.26 to 75.05; $P = 0.037$) (Fig. 4 *C* and *D*).

We were able to collect additional follow-up blood samples in a subset of 19 patients, in which we assessed whether follow-up plasma ctDNA analysis can further improve recurrence prediction. Detection of ctDNA by mqMSP in serial blood samples was associated with poorer RFS (HR, 7.49; 95% CI, 1.62 to 34.63; $P = 0.01$) (Fig. 5*A*). Among seven recurrence patients, four tested positive in postoperative plasma and two additional patients (patients 255 and 485) tested positive in the follow-up plasma samples, indicating that longitudinal ctDNA analysis can increase the sensitivity of predicting recurrence (Fig. 5*B*). In 2 of 12 nonrecurrence patients (patients 215 and 301), positive postoperative plasma ctDNA changed to negative in further follow-up plasma samples after adjuvant chemotherapy, suggesting that adjuvant chemotherapy may have been effective in removing residual tumor load (Fig. 5*B*).

Overall, the analysis of ctDNA by mqMSP had a median lead time of 8.0 mo (range, 0 to 12.5 mo) over radiologic imaging analysis for detecting recurrence. More than one-half (11 out of 20) of the patients who experienced recurrence within 3 y of surgery had positive detection of ctDNA within 2 wk after surgery.

Comparison of Postoperative ctDNA and CEA for Predicting Recurrence. The relationships among postoperative ctDNA status, postoperative CEA level, and recurrence status are shown in *SI Appendix*, Table S5 (4 of the 73 patients did not have postoperative CEA data). The CEA level was above the threshold (5 ng/mL) in the postoperative blood of 4 of the 17 (23.5%) recurrence patients and in 2 of the 52 (3.8%) nonrecurrence patients. While CEA has superior specificity, it suffers from poor sensitivity compared with the ctDNA mqMSP assay (23.5% vs. 55%). Of the 20 recurrence patients, 11 tested positive by mqMSP in postoperative plasma samples.

Parallel Analysis of ctDNA by mqMSP for DNA Methylation Markers and Targeted NGS for Somatic Mutations. Somatic mutation detection by targeted NGS is often used for ctDNA detection. We compared the mqMSP assay with a targeted NGS assay covering 532 cancer-related genes by analyzing a trio of preoperative, postoperative and recurrence plasmas from six recurrence patients. The raw sequencing depth for plasma DNA samples was 14,700× on average. The corresponding tumor tissue samples were analyzed by the same panel, at a raw sequencing depth of ~1,800×, to confirm the somatic mutation results.
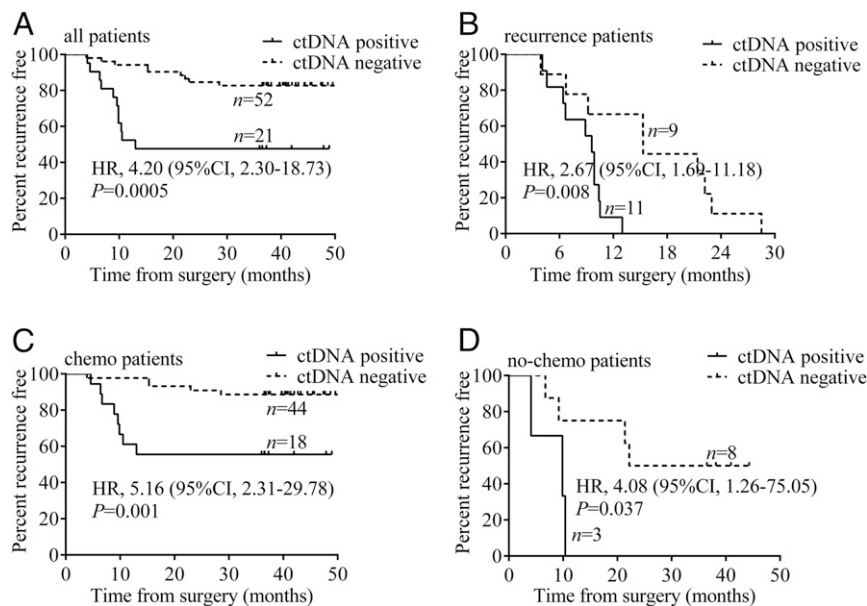

Fig. 4. Postoperative ctDNA status determined by the mqMSP assay predicts recurrence. (*A*) Kaplan–Meier estimates of RFS according to postoperative plasma ctDNA status in 73 patients with positive preoperative plasma ctDNA. (*B*) Kaplan–Meier estimates of RFS according to postoperative plasma ctDNA status for the 20 patients with recurrence. (*C*) Kaplan–Meier estimates of RFS for the 62 patients who received adjuvant chemotherapy. (*D*) Kaplan–Meier estimates of RFS for the 11 patients who did not receive adjuvant chemotherapy. Without adjuvant chemotherapy, all three patients with positive postoperative ctDNA recurred within 12 mo. *P* values were determined by the log-rank test.
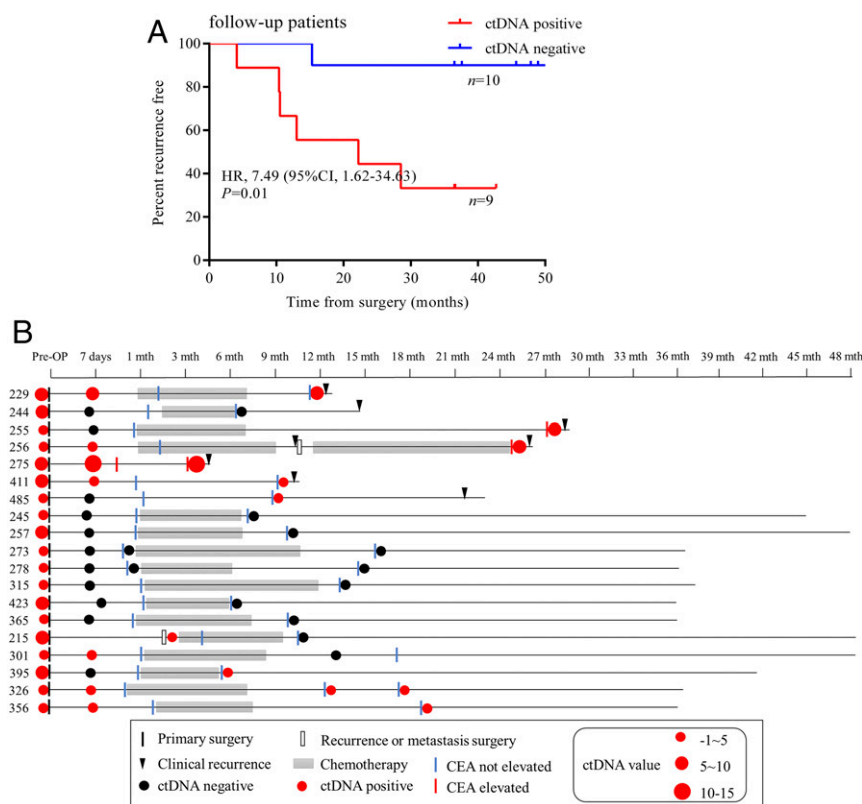
Jin et al.
Efficient detection and post-surgical monitoring of colon cancer with a multi-marker DNA methylation liquid biopsy

PNAS | 5 of 8
https://doi.org/10.1073/pnas.2017421118

Fig. 5. Further follow-up ctDNA analysis by the mqMSP assay may improve recurrence prediction. (*A*) Kaplan–Meier estimates of RFS according to the last sampling result for serial plasma ctDNA analysis (*n* = 19). *P* values were determined by the log-rank test. (*B*) Longitudinal ctDNA status in 19 patients with serial plasma samples. Notably, two patients (patients 255 and 485) initially tested negative in the postoperative plasma samples but then tested positive in the follow-up plasma samples, indicating that longitudinal ctDNA analysis may increase the sensitivity of predicting recurrence. Among 12 nonrecurrence patients, 2 patients (patients 215 and 301) had a positive postoperative plasma ctDNA change to negative in further follow-up plasma after adjuvant chemotherapy.

Qualitatively, the targeted NGS and mqMSP assays produced completely concordant results in the preoperative plasma samples (Fig. 6*A*). The targeted NGS assay detected ctDNA in two of the six postoperative plasma samples, while the mqMSP assay detected ctDNA in two additional (four out of six) postoperative plasma samples. Similarly, the mqMSP results were positive in five of the six plasma samples collected at recurrence, while targeted NGS results were positive in four of the six plasma samples. Median variant allele frequencies (VAFs) quantified by the targeted NGS assay were highly correlated with the mqMSP values ($2^{\Delta Cq}$) ($r = 0.827$; $P = 0.002$) in the 11 samples in which both assays yielded positive results (Fig. 6*B*).

Discussion

The minimal invasiveness and ease of longitudinal sampling make liquid biopsies of ctDNA ideal clinical assays wherever the sensitivity is adequate. Liquid biopsies of cancer patients are currently being used in a few settings, such as companion diagnostics for late-stage lung cancer, where ctDNA is usually present at relatively high concentrations. However, due to the low ctDNA concentrations in body fluids and the heterogeneity of tumor somatic mutations, NGS methods are required to interrogate many tumor DNA markers, making potential clinical tests too expensive and complex for all patients. In addition, due to clonal hematopoiesis and errors derived from library preparation and sequencing, extensive research and sophisticated bioinformatics tools are necessary to reduce false-positive signals (26–28). Such methods might not be easily applicable in clinical settings and certainly are not generally affordable.

To overcome these limitations, we developed the mqMSP assay for quantifying the overall methylation level of 10 subregions in the *SEPT9* gene. In three independent CRC cohorts, this mqMSP assay achieved consistently high sensitivity for detecting ctDNA in plasma samples. In the longitudinal cohort, the assay detected 73 out of the 82 (89.0%) CRC patients, suggesting that it may be generally applicable for postsurgery follow-up. Analysis of postoperative plasma samples collected within 2 wk after surgery by mqMSP was able to identify 11 of the 20 patients who recurred within 3 y, suggesting that a simple qPCR assay performed soon after surgery may be indicative of patient prognosis.

We compared our mqMSP assay with a targeted NGS covering 532 cancer-related genes for plasma ctDNA detection. While the two methods produced quantitatively concordant results for preoperative plasma samples, the mqMSP assay was more sensitive in postoperative and follow-up plasma samples. The lengthy library preparation process is known to lose some DNA sequences, otherwise known as incomplete conversion of input DNA molecules to sequencing data. The sensitivity of the targeted NGS approaches may be improved by increasing sequencing depth at a greater cost. Alternatively, the tumor tissue of each patient can be analyzed first by whole-genome sequencing to identify patient-specific mutation profiles. Subsequently, whole-genome sequencing is performed on postoperative plasma DNA to identify residual disease (28).

There are several limitations in this study. First, we were able to collect follow-up blood samples in only a small subset of the longitudinal cohort. This may partially explain why we failed to detect ctDNA in seven patients with recurrence. For these seven

A

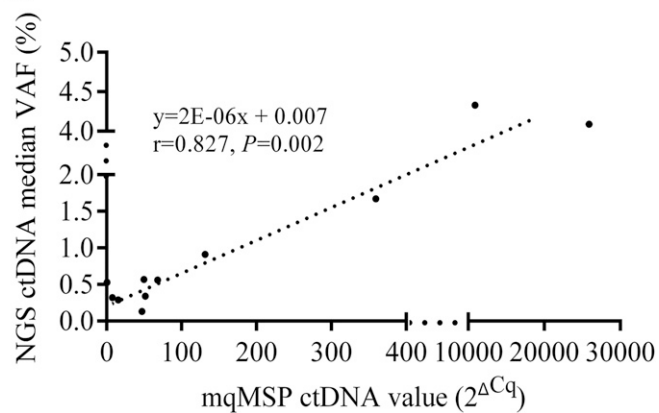| Patient ID	Pre-op ctDNA		Post-op ctDNA		Recurrence ctDNA	
	mqMSP	NGS	mqMSP	NGS	mqMSP	NGS
229	+	+	+	+	+	+
255	+	+	−	−	+	+
256	+	+	+	−	+	+
328	−	−	−	−	−	−
411	+	+	+	−	+	−
667	+	+	+	+	+	+

B

Fig. 6. Comparison of the mqMSP and targeted NGS assays for ctDNA detection and quantification. (*A*) Comparison between the targeted NGS assay and the mqMSP assay in detecting ctDNA for six recurrence patients with preoperative, postoperative, and at recurrence plasma samples. The + and − boxes represent positive and negative detection of ctDNA, respectively. (*B*) Correlation between median VAFs of ctDNA measured by targeted NGS and ctDNA methylation levels measured by mqMSP.

patients, the last blood collected was 7 to 22 mo (median, 12 mo) before recurrence. Second, we observed a false-positive rate of 16.7% in healthy controls, suggesting that the mqMSP assay needs further optimization before it can be used for early CRC screening. We also observed positive ctDNA detection by mqMSP in postoperative plasma from nonrecurrence patients, although in two of these patients, ctDNA could not be detected after adjuvant therapy. Third, a small percentage of CRC patients had false-negative preoperative mqMSP results, which may reflect unusual methylation patterns. Other methods that do not rely on methylation, such as small-panel targeted NGS, may be able to complement mqMSP.

In summary, we have developed an easily implementable and affordable (~$10 consumable cost) mqMSP assay for monitoring recurrence in CRC patients after surgery. Additional research should be done to evaluate whether the current mqMSP assay can be further improved to increase the benefit to CRC patients.

Materials and Methods

Patient Recruitment and Sample Collection. This study was approved by the Ethics Committee of the First Affiliated Hospital of Wenzhou Medical University. All patients involved in the study provided signed informed consent. All patients were confirmed by colonoscopy and pathologic examination when necessary. Advanced adenoma was defined as adenoma with either of the following characteristics: ≥10 mm in size or high-grade dysplasia. Healthy controls were selected based on the absence of hyperplastic and adenomatous polyps by colonoscopy (but allowing benign conditions, such as diverticular disease and hemorrhoids).

Preoperative blood samples were collected before surgery. Postoperative blood samples were collected within 1 to 14 d after surgery. Additional blood samples were collected at follow-up. Blood samples were collected into EDTA tubes and processed to obtain plasma as previously described (29). Tissue samples were collected within 1 h after surgery, snap-frozen, and stored in liquid nitrogen.

DNA Extraction and Bisulfite Conversion. For tissue and buffy coat samples, genomic DNA was extracted with the QIAamp DNA Mini Kit and the QIAamp DNA Blood Mini Kit (QIAGEN), respectively, according to the manufacturer's instructions. Plasma DNA extraction was performed using 2 to 5 mL of plasma with the Apostle MiniMax High-Efficiency cfDNA Isolation Kit, according to the product manual. DNA concentration was measured with a Qubit 3.0 fluorometer (Thermo Fisher Scientific). The extracted DNA was stored at −80 °C until use.

For bisulfite conversion, 1 μg of tissue DNA or 5 to 20 ng of plasma DNA were converted with the Zymo EZ DNA Methylation-Gold Kit according to the manufacturer's instructions.

Tissue and Buffy Coat DNA Methylation Analysis. All primers and probes were synthesized by Tsingke Biological Technology. For bisulfite cloning and sequencing, PCR primers were designed using MethPrimer with human curation when necessary (*SI Appendix*, Table S1). The PCR products were TA-cloned into pGEM-T Easy vector (Promega) for cloning and sequencing.

For qMSP reactions, each subregion of the *SEPT9* gene was analyzed together with the *β-actin* (*ACTB*) control assay in the same reaction using FAM- and VIC-based probes, respectively. PCR amplification was performed in duplicate for each sample.

The KAPA PROBE FAST qPCR Master Mix (2×) Kit was used for qPCR. The reactions were performed in a 25-μL final volume system with 12.5 μL of 2× Master Mix, 0.25 μM of each target primer, 0.1 μM of each target probe, 0.06 μM of each *ACTB* primer, 0.05 μM of *ACTB* probe, and 10 to 20 ng of bisulfite-converted DNA. PCR cycling conditions were as follows: heat activation at 95 °C for 3 min, followed by 45 cycles of 95 °C for 3 s and 60 °C for 30 s, and then 72 °C for 30 s. Primer and probe sequences of the qMSP assays are listed in *SI Appendix*, Table S2.

ctDNA Methylation Analysis. The mqMSP assay contains 10 qMSP assays targeting subregions of the *SEPT9* gene with the FAM-based probes and the same *ACTB* control assay with the VIC-based probe as above.

Positive and negative control reactions were performed with each batch of plasma samples. The positive control reaction contained DNA prepared from the HCT-15 cell line (Cell Bank of the Chinese Academy of Sciences), while the negative control reaction contained DNA prepared from buffy coats of healthy volunteers.

For plasma DNA samples, mqMSP analysis was performed after bisulfite conversion. Plasma DNA samples with inadequate DNA (<5 ng) were excluded. A plasma sample was deemed positive if ΔCq (VIC_{mean} − FAM_{mean}) >−1 and negative if ΔCq (VIC_{mean} − FAM_{mean}) ≤−1. VIC_{mean} and FAM_{mean} values were calculated from the two duplicate reactions of each sample.

Targeted NGS. Targeted NGS was performed on six recurrence patients with serial plasma samples. The KAPA LTP Library Preparation Kit was used to prepare DNA libraries for tumor and paired normal tissues. The Lotus DNA Library Prep Kit (Integrated DNA Technologies) was used to prepare plasma DNA libraries, with dual unique molecular identifiers to minimize false-positives. Subsequently, the xGen Pan-Cancer Panel v2.4 (Integrated DNA Technologies) targeting 532 cancer-related genes was used for target region enrichment, and the enriched libraries were then sequenced on the Illumina HiSeq X NGS platform according to the manufacturer's instructions. Sequencing data analysis was performed with a combination of Fgbio, Burrows–Wheeler Aligner, Genome Analysis Toolkit, and MuTect2. All sequencing files are available from the National Center for Biotechnology Information's BioProject database (accession no. PRJNA687345).

Statistical Analysis. Patient follow-up data were collected between January 26, 2016, and June 8, 2020. Patients without recurrence were followed for 36 to 50 mo. Survival analyses were performed by the Kaplan–Meier method (log-rank) using GraphPad Prism version 6.0. Fisher's least significant difference was used to compare methylation levels among different sample groups. Fisher's exact test was used to compare sensitivities between the mqMSP and SEPT9 assays. Spearman's rank correlation analysis was used to compare the median VAFs quantified by the targeted NGS assay and mqMSP values.

Jin et al.
Efficient detection and post-surgical monitoring of colon cancer with a multi-marker DNA methylation liquid biopsy

www.manaraa.com

1. F. Bray *et al.*, Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. *CA Cancer J. Clin.* **68**, 394–424 (2018).
2. H. J. Schmoll *et al.*, ESMO consensus guidelines for management of patients with colon and rectal cancer. A personalized approach to clinical decision making. *Ann. Oncol.* **23**, 2479–2516 (2012).
3. R. A. Snyder *et al.*; Alliance for Clinical Trials in Oncology Network Cancer Surveillance Optimization Working Group, Association between intensity of posttreatment surveillance testing and detection of recurrence in patients with colorectal cancer. *JAMA* **319**, 2104–2115 (2018).
4. J. A. Meyerhardt *et al.*; American Society of Clinical Oncology, Follow-up care, surveillance protocol, and secondary prevention measures for survivors of colorectal cancer: American Society of Clinical Oncology clinical practice guideline endorsement. *J. Clin. Oncol.* **31**, 4465–4470 (2013).
5. B. D. Nicholson *et al.*, Blood CEA levels for detecting recurrent colorectal cancer. *Cochrane Database Syst. Rev.* **2015**, CD011134 (2015).
6. M. Bretthauer *et al.*; Nordic-European Initiative on Colorectal Cancer (NordICC) Study Group, Population-based colonoscopy screening for colorectal cancer: A randomized clinical trial. *JAMA Intern. Med.* **176**, 894–902 (2016).
7. J. C. M. Wan *et al.*, Liquid biopsies come of age: Towards implementation of circulating tumour DNA. *Nat. Rev. Cancer* **17**, 223–238 (2017).
8. G. Siravegna, S. Marsoni, S. Siena, A. Bardelli, Integrating liquid biopsies into the management of cancer. *Nat. Rev. Clin. Oncol.* **14**, 531–548 (2017).
9. H. Osumi, E. Shinozaki, K. Yamaguchi, H. Zembutsu, Clinical utility of circulating tumor DNA for colorectal cancer. *Cancer Sci.* **110**, 1148–1155 (2019).
10. Z. Wang *et al.*, Detection of EGFR mutations in plasma circulating tumour DNA as a selection criterion for first-line gefitinib treatment in patients with advanced lung adenocarcinoma (BENEFIT): A phase 2, single-arm, multicentre clinical trial. *Lancet Respir. Med.* **6**, 681–690 (2018).
11. N. Tarazona *et al.*, Targeted next-generation sequencing of circulating-tumor DNA for tracking minimal residual disease in localized colon cancer. *Ann. Oncol.* **30**, 1804–1812 (2019).
12. J. Tie *et al.*, Circulating tumor DNA analysis detects minimal residual disease and predicts recurrence in patients with stage II colon cancer. *Sci. Transl. Med.* **8**, 346ra92 (2016).
13. J. Tie *et al.*, Serial circulating tumour DNA analysis during multimodality treatment of locally advanced rectal cancer: A prospective biomarker study. *Gut* **68**, 663–671 (2019).
14. T. Reinert *et al.*, Analysis of plasma cell-free DNA by ultradeep sequencing in patients with stages I to III colorectal cancer. *JAMA Oncol.* **5**, 1124–1131 (2019).
15. J. D. Warren *et al.*, Septin 9 methylated DNA is a sensitive and specific blood test for colorectal cancer. *BMC Med.* **9**, 133 (2011).
16. R. Pidsley, C. Stirzaker, "Cancer methylation biomarkers in circulating cell-free DNA" in *Clinical Epigenetics*, L. Hesson, A. Pritchard, Eds. (Springer, 2019), pp. 217–245.
17. V. Constâncio, S. P. Nunes, R. Henrique, C. Jerónimo, DNA methylation-based testing in liquid biopsies as detection and prognostic biomarkers for the four major cancer types. *Cells* **9**, 624 (2020).
18. T. R. Church *et al.*; PRESEPT Clinical Study Steering Committee, Investigators and Study Team, Prospective evaluation of methylated SEPT9 in plasma for detection of asymptomatic colorectal cancer. *Gut* **63**, 317–325 (2014).
19. C. Tham *et al.*, Postoperative serum methylation levels of TAC1 and SEPT9 are independent predictors of recurrence and survival of patients with colorectal cancer. *Cancer* **120**, 3131–3141 (2014).
20. S. Y. Shen *et al.*, Sensitive tumour detection and classification using plasma cell-free DNA methylomes. *Nature* **563**, 579–583 (2018).
21. H. Luo *et al.*, Circulating tumor DNA methylation profiles enable early diagnosis, prognosis prediction, and screening for colorectal cancer. *Sci. Transl. Med.* **12**, eaax7533 (2020).Correction in: *Sci. Transl. Med.* **12**, eabc1078 (2020).
22. S. Guo *et al.*, Identification of methylation haplotype blocks aids in deconvolution of heterogeneous tissue samples and tumor tissue-of-origin mapping from plasma DNA. *Nat. Genet.* **49**, 635–642 (2017).
23. X. Chen *et al.*, Non-invasive early detection of cancer four years before conventional diagnosis using a blood test. *Nat. Commun.* **11**, 3475 (2020).
24. M. C. Liu, Sensitive and specific multi-cancer detection and localization using methylation signatures in cell-free DNA. *Ann. Oncol.* **31**, 745–759 (2020).
25. T. deVos *et al.*, Circulating methylated SEPT9 DNA in plasma is a biomarker for colorectal cancer. *Clin. Chem.* **55**, 1337–1346 (2009).
26. J. J. Chabon *et al.*, Integrating genomic features for non-invasive early lung cancer detection. *Nature* **580**, 245–251 (2020).
27. P. Razavi *et al.*, High-intensity sequencing reveals the sources of plasma circulating cell-free DNA variants. *Nat. Med.* **25**, 1928–1937 (2019).
28. A. Zviran *et al.*, Genome-wide cell-free DNA mutational integration enables ultra-sensitive cancer monitoring. *Nat. Med.* **26**, 1114–1124 (2020).
29. Z. Yang *et al.*, Dynamics of plasma EGFR T790M mutation in advanced NSCLC: A multicenter study. *Target. Oncol.* **14**, 719–728 (2019).

8 of 8 | **PNAS**
https://doi.org/10.1073/pnas.2017421118

Jin et al.
Efficient detection and post-surgical monitoring of colon cancer with a multi-marker DNA