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RAPID COMMUNICATIONS

# Stool Methylated DNA Markers Decrease Following Colorectal Cancer Resection—Implications for Surveillance

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**Abstract**
*Background* Molecular changes associated with colorectal cancer (CRC) are detected by stool deoxyribonucleic acid testing but could persist following tumor resection.
*Aims* We sought to determine whether methylated gene markers in stool normalize after CRC resection.
*Methods* We studied stools from 22 CRC cases before and after subtotal resection and from 80 colonoscopy-normal controls. In blinded fashion, target genes (methylated *NDRG4* and *BMP3*) were captured from stool supernatant, bisulfite-treated, and assayed by quantitative allele-specific real-time target and signal amplification. Results were dichotomized at 95 % specificity cutoffs.
*Results* Among CRC cases, median methylated *NDRG4* and *BMP3* levels decreased dramatically (4- to 15-fold) following resection, $p = 0.003$ and $p < 0.0001$, respectively. Among the 14 cases with elevated preoperative levels, 13 (93 %) fell into the normal range after surgery, $p = 0.0002$. A case whose stool methylated *NDRG4* level increased sharply after surgery was found to have recurrent CRC.
*Conclusions* Methylated gene marker levels clear from stool following CRC resection unless disease is present. Postoperative stool marker levels are informative and may be of value in surveillance.

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**Abbreviations**

| | |
|---|---|
| AJCC | American Joint Committee on Cancer |
| CRC | Colorectal cancer |
| DNA | Deoxyribonucleic acid |
| Ln | Natural logarithm |

## Introduction

Stool DNA testing has emerged as a noninvasive approach to colorectal cancer (CRC) screening [1, 2]. As expanded applications for this technology are under consideration, biological and clinical data are needed to guide rational decision making. Of fundamental importance to the potential use of stool DNA testing for postoperative CRC surveillance is an understanding of what happens to stool marker levels after tumor resection.

It is well established that both genetic and epigenetic abnormalities found in CRC may also occur in the histologically normal surrounding mucosa [3]. These "field changes" may not be completely abolished with CRC resection, the extent of which is determined by vascular anatomy and lymphatic drainage rather than molecular mucosal margins [4]. Remaining field changes may also play a role in the development of both synchronous and metachronous CRC [5]. It is not known how commonly molecular field changes persist following CRC resection or if they would cause elevated stool DNA levels in the absence of gross neoplasia.

Next-generation stool DNA testing, using optimized assays that target a multi-marker panel of methylated genes (*BMP3* and *NDRG4*), mutant *KRAS* and hemoglobin, has

proven highly sensitive and specific for both CRC and advanced precancers [6–8]. While gene mutations have been shown to clear from stool following neoplasm resection [9], changes in methylation markers have not to our knowledge been evaluated. If marker levels normalize following CRC resection, then it would be justified to explore stool DNA as a potentially accurate and cost-effective tool for postoperative surveillance, where >150 colonoscopies are currently needed to find a single metachronous CRC [5]. Thus, we aimed in this study to compare stool levels of methylated *BMP3* and *NDRG4* from CRC patients before and after subtotal colectomy.

## Materials and Methods

### Patients and Sample Collection

After approval from the Institutional Review Board (Mayo Clinic, Rochester MN), we recruited CRC patients on whom preoperative stools were archived. Patients consented to submit a second stool ≥6 months after surgery. All patients were required to be in compliance with postoperative colonoscopic surveillance, which was scheduled in accordance with practice guidelines [5]. Case patients were allowed to submit stool specimens in the interval between surveillance colonoscopies. To avoid stool assay artifacts caused by bowel purgatives, samples had to be submitted either prior to colonoscopy prep or at least 1 week after the procedure. All cases were followed forward in the medical record for a minimum of 18 months after study participation.

To set marker cutoffs and to avoid analytical bias, stools from colonoscopy-normal controls were independently selected from our freezer archive and frequency matched on age (±10 years) and sex. All stools in this study were collected in preservative buffer, homogenized and aliquoted upon receipt, and frozen at -80 C until assayed in a single batch by technicians blinded to clinical data.

### Analytical Techniques

#### *DNA Sequence-Specific Capture and Bisulfite Treatment*

A 2-g equivalent of stool supernatant was used for multiplex hybrid capture of gene targets (*β-actin*, *BMP3*, and *NDRG4*) and bisulfite-treated with EZ DNA Methylation Kit (Zymo Research).

#### *Assay of Target Genes*

Deoxyribonucleic acid (DNA) markers were quantified by the Quantitative Allele-specific Real-time Target and Signal amplification (QuARTS) method as previously described [10]. This technique combines a polymerase-based DNA amplification of target gene sequences with invasive cleavage-based signal amplification. An unmethylated region of the human *β-actin* gene was also amplified to ensure sufficient fecal DNA recovery. Additional method details and primer sequences for all reactions have been previously published [8, 11].

**Table 1** Patient and tumor characteristics

| | CRC cases ($n = 22$) | Controls ($n = 80$) |
|---|---|---|
| Patients | | |
| Median age, years (range) | 59 (38–88) | 68 (41–84)[c] |
| Male (%) | 54 | 54 |
| Surveillance complete (%)[a] | 22 (100) | – |
| Median follow-up, months (range)[b] | 19 (6–46) | – |
| Colorectal cancer | | |
| Proximal to splenic flexure (%) | 8 (36) | – |
| Rectal (%) | 9 (41) | – |
| Median size, cm (range) | 3.1 (0.8–9.2) | – |
| AJCC (2010) stage (%) | | – |
| I | 6 (27) | – |
| IIA | 6 (27) | – |
| IIIA | 3 (14) | – |
| IIIB | 7 (32) | – |

[a] By 1- or 3-year postoperative surveillance colonoscopy

[b] Time elapsed between surgery and postoperative stool collection

[c] $p = 0.15$

### Statistical Analysis

The primary study outcome was the proportion of discordant tests among CRC patients before and after surgery, assessed by a two-sided McNemar's test. We estimated that a minimum of 10 discordant pre- versus post-pairs of CRC cases would provide greater than 80 % power to demonstrate a difference in the proportion of pre- and postoperative stool marker results of 0.9, compared to a null hypothesis of 0.5 at the 0.05 significance level. Copy numbers of methylated *BMP3* and *NDRG4* were assayed from preoperative CRC patients and controls and then compared using the Wilcoxon rank sum test. Marker level results were then dichotomized (positive vs. negative) based on 95 % specificity cutoffs from the comparison of preoperative CRC case to control results. This cutoff value was then used to determine the postoperative test result for CRC cases. Quantitative differences between marker levels before and after surgery in the CRC cases were compared using the Wilcoxon signed-rank test for matched pairs. Analyses were performed using JMP version 9.0.1 (SAS Institute, Cary, NC).

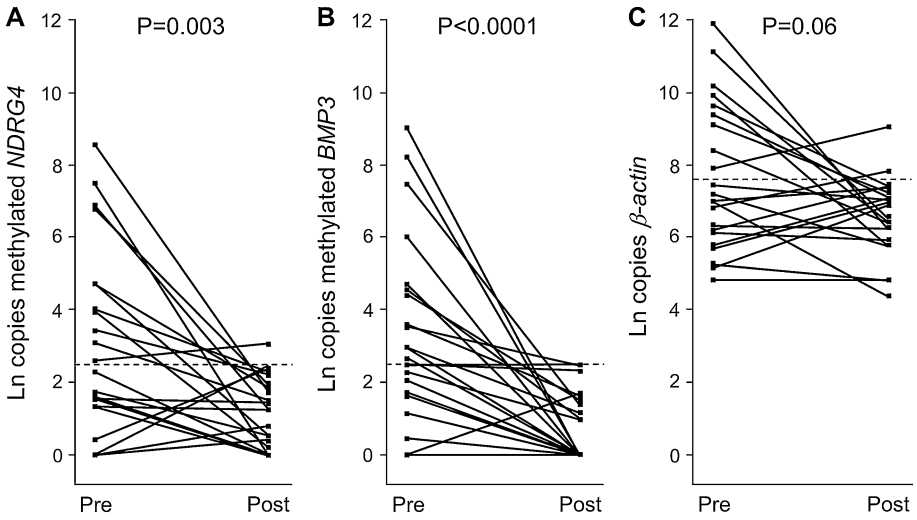


**Fig. 1** Natural logarithm (Ln)-transformed marker copies, pre- versus postoperatively for **a** methylated *NDRG4*; **b** methylated *BMP3*; and **c** *β-actin*. *p* values are by Wilcoxon signed-rank test for matched pairs. Reference lines are 95 % specificity cutoffs for preoperative CRC cases compared to colonoscopy-negative normal controls

## Results

A recruitment letter was sent to 45 eligible CRC patients with preoperative stools in our archive. Of those, 22 cases consented to submit a postoperative specimen. From our freezer archive, samples from 80 colonoscopy-normal control patients, matched on age and sex, were independently selected for study. Patient characteristics are presented in Table 1.

The median (inter-quartile range) copy number of methylated *BMP3* per 2 g stool was 16 (4–95) among preoperative CRC patients and 0 (0–5) among controls ($p < 0.001$). The median copy number of methylated *NDRG4* per 2 g stool was 11 (3–112) and 0 (0–4) among CRC patients and controls, respectively ($p < 0.001$).

Among the 14 CRC cases with elevated stool marker levels (above 95 % specificity cutoffs), 13 (93, 95 % CI 68–98 %) fell into the normal range following CRC resection, $p = 0.0002$. Figure 1 shows natural logarithm-transformed values for each marker, pre- and postoperatively. In contrast to the highly significant decreases noted with each methylation marker, the decrease in stool levels of *β-actin* did not meet statistical significance. Furthermore, differences in methylation marker levels between stools obtained before and after surgery remained highly significant when corrected by *β-actin* levels ($p = 0.001$ for both markers). Three patients had adenomas found within the surgical field of the tumor; all were positive for either *NDRG4* or *BMP3*.

In those with negative stool tests, marker levels remained below the positive test cutoff for the duration of the study. One patient was diagnosed with peritoneal CRC metastasis 6 months after study participation and 3 years after resection of the primary tumor; this individual had a sharp postoperative rise in methylated *NDRG4* but did not meet the test-positive threshold during the study. This recurrence was detected by rising carcinoembryonic antigen (CEA). Nine months after study participation, an elevated carcinoembryonic antigen (30 ng/mL) was observed in the one patient whose *NDRG4* did not normalize after surgery; after 3 years of follow-up, recurrence has not been detected. At surveillance colonoscopy, only 2 patients were found to have adenomatous polyps; these were diminutive (<1 cm) and not detected by either methylation marker.

There was no association between tumor size or clinical stage (I–II vs. IIIA–B) and preoperative methylation test results. Methylated *BMP3* and *NDRG4* test results were not influenced by tumor location (proximal vs. distal to splenic flexure).

## Discussion

We demonstrate that methylated gene markers present in stool from patients with CRC fall back into the normal range following subtotal resection unless pathology is present. CRC per se appears to be the primary source of exfoliated stool DNA markers prior to tumor resection, and our findings represent evidence against a general field cancerization defect as a source of persistent abnormal stool results postoperatively.

Our findings support a potential role for the use of stool DNA testing in surveillance following CRC resection to complement conventional approaches. In the absence of luminal neoplasia on follow-up colonoscopies or of clinical evidence of tumor recurrence, all CRC cases had normal stool marker levels postoperatively. In contrast, *NDRG4* rose sharply in 1 patient later found to peritoneal metastases.

The normalization in stool levels of methylation markers following CRC resection could not be explained by the

overall reduction in mucosal surface area occurring with segmental colon resection. Stool *β-actin* levels, which reflect total gastrointestinal DNA exfoliation, did not significantly decrease after CRC resection. Furthermore, methylation markers adjusted by *β-actin* level also fell significantly in postoperative stools, supporting the likelihood that methylation markers were shed by the CRC tumors rather than a generalized molecular field change.

The present study has several limitations. First, case patients were recruited from a highly motivated population, having already participated in stool DNA research, and may not have been representative of the general population. Second, while our findings are biologically and clinically relevant, the specific performance of methylation markers in our research laboratory is not directly comparable to commercial tests and did not include fecal immunochemical assay for hemoglobin. Third, the experimental design assumes that stool DNA markers remain constant in normal controls over time. While stool methylation markers may increase slightly with age [12], we did not adjust pre- and postoperative comparisons given the relatively short period of time between collections (6–46 months). While the patient population was largely non-Hispanic whites, we have previously shown that levels of methylated DNA markers in stools do not appear to be affected by race [12]. It was not possible to determine whether adenomas adjacent to CRC contribute to stool test accuracy. Preoperative methylated DNA levels did not reach the positive test threshold in a CRC patient with subsequent peritoneal recurrence. While it is conceivable that peritoneal metastases could have invaded the small or large bowel lumen to account for the postoperative rise in stool marker levels, there was not an opportunity to explore this further. In the absence of anatomical continuity with the alimentary tract and trans-serosal invasion, there is limited biological plausibility for the detection of distant recurrences by stool assay of methylated DNA. The primary study comparison benefitted from the use of each patient as their own control, which should minimize bias for most other clinical variables.

Our findings open the door for further consideration of stool DNA testing for surveillance following CRC resection. Larger cohorts of patients under surveillance for CRC recurrence are needed to corroborate and extend our findings. For the application of stool DNA testing to post-polypectomy surveillance, evaluation of molecular marker changes in stool following colonoscopic removal of advanced adenomas and serrated polyps will be important.

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**Conflict of interest** Mayo Clinic has entered into an intellectual property development agreement with Exact Sciences (Madison, WI) whereby inventors (Ms. Yab, Mr. Taylor, Mr. Mahoney, Drs. Kisiel, and Ahlquist) could share in equity or future royalties in accordance with Mayo Clinic policy.

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