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Stool DNA Testing for the Detection of Colorectal Neoplasia in Patients with Inflammatory Bowel Disease

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Summary

Background—Current approaches to the detection of colorectal neoplasia associated with inflammatory bowel disease (IBD-CRN) are suboptimal.

Aim—We tested the feasibility of using stool assay of exfoliated DNA markers to detect IBD-CRN.

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Methods—This investigation comprised tissue and stool studies. In the tissue study, gene sequencing and methylation assays were performed on candidate genes using tissue DNA from 25 IBD-CRNs and from 25 IBD mucosae without CRN. Mutations on *P53, APC, KRAS, BRAF* or *PIK3CA* genes were insufficiently informative, but several aberrantly methylated genes were highly discriminant. In the stool study, we evaluated candidate methylated genes (*vimentin, EYA4, BMP3, NDRG4*) in a prospective blinded study on buffered stools from 19 cases with known IBD-CRN and 35 age- and sex-matched IBD controls without CRN. From stool-extracted DNA, target genes were assayed by quantitative allele-specific real-time target and signal amplification method.

Results—IBD-CRN cases included 17 with ulcerative colitis (UC) and 2 with Crohn's disease (CD); 9 had cancer and 10 had dysplasia. Controls included 25 with UC and 10 with CD. Individually, *BMP3, vimentin, EYA4*, and *NDRG4* markers showed high discrimination in stools with respective areas under the ROC curve of 0.91, 0.91, 0.85, and 0.84 for total IBD-CRN and of 0.97, 0.97, 0.95, and 0.85 for cancer. At 89% specificity, the combination of mBMP3 and mNDRG4 detected 9/9 (100%) of CRC and 80% of dysplasia, 4/4 (100%) of high grade and 4/6 (67%) of low grade.

Conclusion—These findings demonstrate feasibility of stool DNA testing for noninvasively detecting IBD-CRN.

Keywords

Stool DNA; inflammatory bowel disease; cancer surveillance; colorectal neoplasms; DNA methylation

Introduction

Patients with inflammatory bowel disease (IBD) are at increased risk of colorectal neoplasia (CRN), including colorectal cancer (CRC).[1, 2] Factors known to increase CRC risk in IBD include duration and extent of chronic ulcerative colitis (CUC) or Crohn's colitis (CD), presence of primary sclerosing cholangitis (PSC), degree of histological activity, and family history of CRC.[3–6] To reduce CRC risk, patients with IBD undergo periodic surveillance colonoscopies with multiple random biopsies to detect early visible and occult CRN (dysplasia and cancer).[7]

Limitations of this colonoscopic surveillance, as currently practiced, include under-sampling with random biopsies, an unknown ideal frequency for performing the surveillance exam, and low grade of evidence for effectiveness.[8–10] Some centers use image-enhancing techniques such as chromoendoscopy for surveillance. This has the advantage of identifying more dysplastic lesions by targeted rather than random biopsies,[11] but requires special training and sometimes extended endoscopy time. However, regardless of the surveillance technique used, CRN may be missed due to difficulty visualizing neoplastic lesions which are obscured against a background of chronic inflammatory changes.[12, 13] Identifying biomarkers that can provide complementary information to colonoscopy could fill an important clinical need in this patient population.

Stool assay of exfoliated molecular markers represents a noninvasive approach that could serve as such an adjunct to colonoscopy.[14, 15] While next-generation assay methods have yielded high detection rates for both sporadic CRC and pre-cancer,[16–18] stool DNA testing as an approach to neoplasia detection in the IBD population has not been explored.

Tissue-based studies have demonstrated that IBD-CRN is associated with numerous molecular alterations, including acquired mutations in *p53*,[19, 20] *APC*,[21] *K-ras*,[22–24] and

BRAF[25] as well as aberrant methylation in *EYA4*[26], *ER, p16, MYOD, p14, E-cadherin, RUNX3, MINT1* and *COX-2*.[27–31]. Several other genes, such as *BMP3, vimentin (VIM),*[32] *septin 9*[33] and *NDRG4*,[16] are selectively methylated in sporadic CRC but have not been investigated in IBD.

The aims of this investigation were to (1) assess the discriminant value of the mutation markers *p53, APC, BRAF, K-ras* and *PIK3CA* and the methylation markers *VIM, BMP3, EYA4* and *septin 9* for detection of IBD-CRN based on DNA extracted from well-characterized tissue specimens and (2) using the most discriminant tissue markers, prospectively assess the feasibility of stool DNA testing for the detection of premalignant and malignant IBD-CRN.

Methods

Our investigation was approved by the Institutional Review Boards at: Mayo Clinic, Rochester Minnesota, USA; University of Chicago, Chicago Illinois, USA; and Mount Sinai School of Medicine, New York New York, USA.

Tissue Study

Patients—Tissues were identified from a single-center archive of IBD-CRC case and IBD control specimens after confirmation of histologic diagnosis. Cases and controls were matched for age (within a 10-year range), gender, disease duration, anatomic extent (left-sided/extensive) and PSC status (yes/no). DNA was extracted from paraffin-embedded tissues as described.[34]

Mutation Marker Gene Sequencing—Candidate exons on *APC, p53, K-ras, BRAF* and *PIK3CA* were amplified using real-time PCR (see Supplemental Methods).

Real-Time Methylation-Specific PCR (MSP)—After bisulfite treatment, MSP was performed on *VIM, BMP3* and *septin 9* using Taq polymerase (Invitrogen, Carlsbad, CA) and on *EYA4* using SYBR Green master mix (Roche, Mannheim, Germany).

Stool Study

Patients—Case patients with established IBD-CRN were recruited. Those who had undergone endoscopic or surgical treatment of neoplasia or with a history of other neoplasia of the gastrointestinal tract or respiratory system were excluded. Each site recruited IBD control patients undergoing surveillance colonoscopy, matched on age (in 5 year strata) and sex. As anticipated,[35] matching on more variables was not possible during prospective enrollment but data was collected on IBD diagnosis (CD/UC/indeterminate), IBD duration, extent of colitis and PSC. IBD activity was assessed by a single expert pathologist, using a previously published protocol.[36] After informed consent, participants were given a container and toilet seat mounting bracket kit to collect stools prior to or at least one week after colonoscopy or sigmoidoscopy.[37, 38]

Sequence-specific gene capture—A 2-gram equivalent of stool supernatant was used for multiplex capture of gene targets (β- *actin, VIM, EYA4, BMP3* and *NDRG4*) by amino conjugated oligonucleotides complementary to target sequences (see Supplemental Methods).[39]

Assay of Methylated Markers—After capture, target DNA was bisulfite treated and quantitative allele-specific real-time target and signal amplification (QuARTS) reactions were performed on Roche 480 LightCyclers (Indianapolis, IN), as described (see

Supplemental Methods).[16] *EYA4* methylation was assayed by methylation specific PCR, performed on a LightCycler 480 using SYBR Green I Master (Roche) as described.[40]

Statistical Analysis

Based on a comparison of immunochemical fecal occult blood testing against colonoscopy for the detection of sporadic CRN,[41] the feasibility for IBD-CRN detection by stool DNA testing at this initial phase of evaluation was defined *a priori* as sensitivity for neoplasia >40%. Based on conservative pre-study assumptions, it was estimated that 15 patients in the case group would provide 80% power to distinguish a true sensitivity of 70% from a null value of 40% with a 1-sided one sample proportion test at the 5% level. The distributions of each marker as a continuous variable were compared between cases and controls using the Wilcoxon rank-sum test (JMP v8.0, SAS Institute, Cary NC, USA). Logistic regression was used to calculate receiver operating characteristics (ROC) curves, from which specificity cut-offs were imputed and marker sensitivities (with 95% confidence intervals (CI)) were calculated. To further study the effects of known prognostic factors on marker levels, differences in baseline variables between cases and controls were tested by Chi-square for proportions and Wilcoxon rank-sum for continuous data. When baseline variables were significantly different, case and control marker results were stratified to assess confounding. Univariate and multivariate logistic regression models assessed potential interaction by age, sex and clinical risk factors, including comorbid PSC (yes/no), disease duration (in years) and disease extent (left-sided/extensive). ANOVA was used to assess possible associations between IBD activity (inactive/mild/moderate/severe) and marker levels.

Results

Tissue Study

Clinical characteristics were well-matched between cases and controls (Table 1). Figure 1 summarizes the results of DNA sequencing for the case samples. Across 6 *APC* regions overlapping the mutation cluster region (1, 2, C, N, Y, L2), only 3 mutations were found. Four mutations were found on *K-ras*. As anticipated, *p53* was the most informative marker with 11 mutations detected; however, these were spread out across a wide range of sites on all 5 exons tested. No mutations were identified on *BRAF* or *PIK3CA*. While specificity was 100% (no mutations found among control tissues), aggregate sensitivity using all 14 mutation markers combined was only 60%. This is similar to observed rates of DNA mutations assayed from tissues of sporadic CRC and advanced adenomas.[42]

For each of the methylation markers, ROC curves were constructed. Areas under the curve (AUC) were 0.97, 0.87, 0.81 and 0.73 for methylated *EYA4 (mEYA4)*, *VIM (mVIM)*, *BMP3 (mBMP3)* and *Septin 9* respectively. Thus, *mEYA4*, *mVIM* and *mBMP3* were selected for stool DNA testing. In addition, methylated *NDGR4 (mNDRG4)* was also selected because of its high discrimination for sporadic CRN in studies performed after the completion of the tissue study.[16]

Stool Study

Given the high discrimination observed with methylation markers in the tissue study, an analysis of stool from independent sets of cases and controls was performed. Between January 1, 2009 and October 31, 2011, a total of 23 eligible cases and 220 eligible controls were identified and contacted. Nineteen IBD case patients with biopsy-confirmed CRN and 35 IBD control patients without CRN submitted stools (Table 2). Although the proportions of IBD diagnoses and comorbid PSC were not significantly different between the two groups, cases had significantly longer disease duration (p=0.0008) and were significantly

more likely to have extensive disease involvement (p=0.01). There was no difference in disease activity between cases and controls (p=0.44).

Case neoplasms included 9 cancers with a median size of 2.3 cm (range 0.8 – 5 cm). Six of the 9 (67%) were proximal to the splenic flexure. Median stage[43] was I (range I to IIIC). Additional neoplasms included 8 discrete polypoid dysplastic lesions (3 high-grade dysplasia [HGD], 5 low-grade dysplasia [LGD]) with a median size of 2.3 cm (range 1.0 – 6.2) and two flat lesions (1 HGD, 1 LGD) detected on random biopsy (size unknown).

β-*actin*, a marker of human DNA recovery, amplified in all case and control samples; therefore all patients were included in the analysis. All 4 markers individually showed high discrimination for cancer (Figure 2). AUCs with *mBMP3*, *mVIM*, *mEYA4* and *mNDRG4* were 0.97, 0.97, 0.95 and 0.85, respectively. For IBD-CRN the AUC with *mBMP3*, *mVIM*, *mEYA4* and *mNDRG4* were 0.91, 0.91, 0.85 and 0.84, respectively. For dysplasia, the AUC with *mBMP3*, *mVIM*, *mEYA4* and *mNDRG4* was 0.84, 0.85, 0.75 and 0.77, respectively. Stool assay of *mBMP3* alone at 91% specificity was 100% (9/9) sensitive for CRC, 70% (7/10) of dysplasia, and 84% (16/19) sensitive for all CRN (Table 3). At 89% specificity, the combination of mBMP3 and mNDRG4 detected 9/9 (100%) of CRC and 80% of dysplasia, 4/4 (100%) of high grade and 4/6 (67%) of low grade.

The dynamic range of methylated copy numbers between cases and controls was wide for each stool marker (Figure 3). Among cases, copy numbers of *mBMP3*, *mVIM*, *mEYA4* or *mNDRG4* were not significantly different for proximal versus distal neoplasms (p = 0.58, 0.73, 0.83 and 0.85, respectively). After stratifying for case verus control status, marker levels were not significantly different when comparing patients with CUC and Crohn's disease.

In multivariate analyses, methylation markers for CRN detection remained significant in models which included age, sex, disease duration, disease extent or the presence of PSC (Supplemental Table 1). ANOVA did not demonstrate any association between markers and disease activity for either cases or controls. Disease duration showed weak correlation with marker levels by univariate linear regression; however, when stratified by case and control status, this association was no longer significant for any of the four methylation markers evaluated. Furthermore, there was no association between anatomic extent of disease (extensive vs. left-sided) and marker levels for either cases or controls.

Discussion

Using DNA methylation markers which were discriminant in tissue, we found that the stool assay achieved high detection rates of both CRC and dysplasia in IBD patients. For example, stool assay of a single informative marker, mBMP3, detected 100% of CRC and 84% of all neoplasms at 91% specificity. Importantly, stool marker levels assayed were unaffected by neoplasm site within the colorectum, as we have observed with sporadic colorectal neoplasia.[16] These early results surpassed our predetermined threshold for feasibility.

While corroborative studies are clearly needed, our data suggest the potential usefulness of stool DNA testing to inform the frequency and rigor of colonoscopic surveillance. A non-invasive test which could be performed without bowel cleansing in a patient's own home might improve compliance with surveillance, which is currently poor, even among high-risk patients.[44, 45] Algorithms incorporating stool DNA as a complement to colonoscopy could potentially lengthen the interval between surveillance examinations in marker-negative patients, which could also reduce the high cost of surveillance endoscopy.[46] Conversely, a

patient with a positive stool DNA test may benefit from colonoscopy at shorter surveillance intervals and/or using enhanced imaging techniques such as chromoendoscopy.

The tissue study based on well-matched cases and controls showed that methylation markers are highly discriminant for IBD-CRN. These tissue findings corroborate our previous observations with *mBMP3* and *mEYA4*.[26]

Prior studies of methylation markers in IBD-CRN have focused on tumor suppressor genes.[27, 29, 30, 47, 48] While *BMP3* and *NDRG4* are known tumor suppressors in CRC,[16, 49, 50] the biology need not be fully understood before a marker is clinically useful. The roles of *EYA4* and *VIM* in IBD-carcinogenesis are unclear, and these genes are aberrantly methylated in other tissues as well.[51–53] Studies have also demonstrated that *mVIM* is a sensitive stool marker for sporadic CRC.[54, 55]

Our study has several limitations. First, this was a case-control study sized to assess early feasibility of stool DNA testing for detection of IBD-CRN. While sample size was sufficient to meet the central aim, the power to evaluate sub-classes among covariates and marker combinations was limited. Larger studies are needed to achieve greater precision for the sensitivity estimates. Additionally, only 2 endoscopically inapparent (flat) dysplastic lesions were available for analysis, limiting inferences that can be drawn about discrimination for this endpoint. Nevertheless, the high sensitivity achieved by the combination of *mBMP3* and *mNDRG4* warrants further study, particularly when considering that these two markers have proved complementary for detection of sporadic CRN in studies with large sample sizes.[16–18] Methylated *BMP3* detected more neoplasms in stool compared to tissue, which could reflect a high prevalence of sporadic-type CRN in IBD patients.[56] Second, while cases and controls in the stool study were well-matched on most variables, cases had a longer median duration of IBD and were more likely to have extensive disease. Accordingly, disease duration was further evaluated in stratified comparisons and multivariate models and was not found to significantly influence marker levels. Other parameters of disease severity, including anatomic extent of inflammation, degree of inflammation, and presence of concomitant PSC, also had no effect on stool marker levels. Last, conventional colonoscopy with non-targeted biopsies was used as the criterion standard, and this approach lacks sensitivity for CRN as currently practiced for IBD surveillance.[12, 13] Control patients who tested positive for methylation markers might therefore have had falsely negative colonoscopies, which would have affected specificity estimates. Underscoring the imperfect nature of colonoscopy, two of the 9 CRC cases with positive stool results in this study were missed on colonoscopy and diagnosed only after colectomy.

These early results in tissue and stool represent an important first step in the evaluation of stool DNA as a noninvasive tool for detection of CRN in IBD patients. Further studies are needed to corroborate and expand these novel findings. Particularly, prospective cohort studies conducted in the IBD surveillance setting will help determine how this noninvasive tool might improve colonoscopy yield and patient outcomes, and potentially lower healthcare costs.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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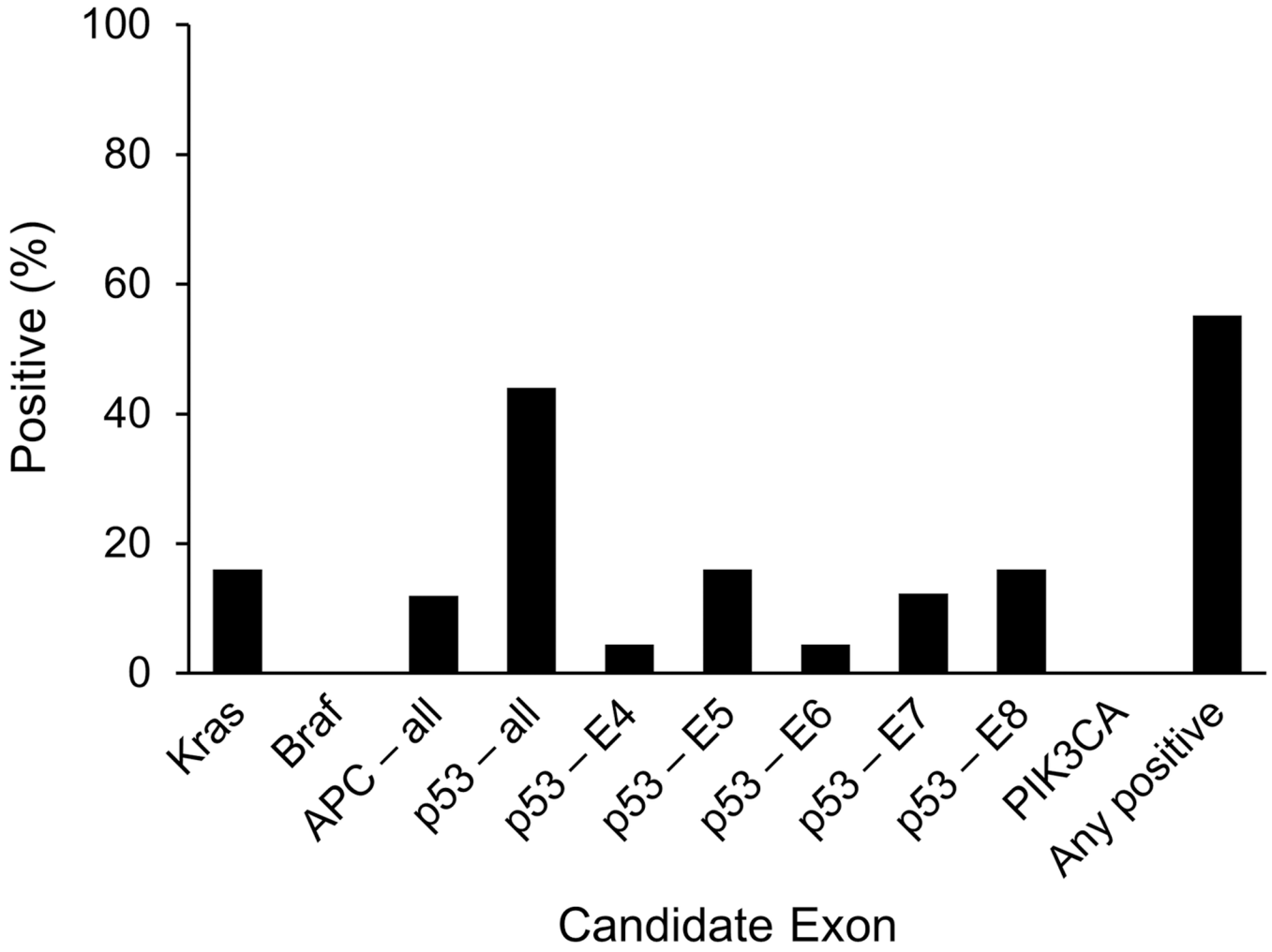

Figure 1.
Gene Mutations Detected in Tissue DNA from Inflammatory Bowel Disease Associated Cancers (n=25)

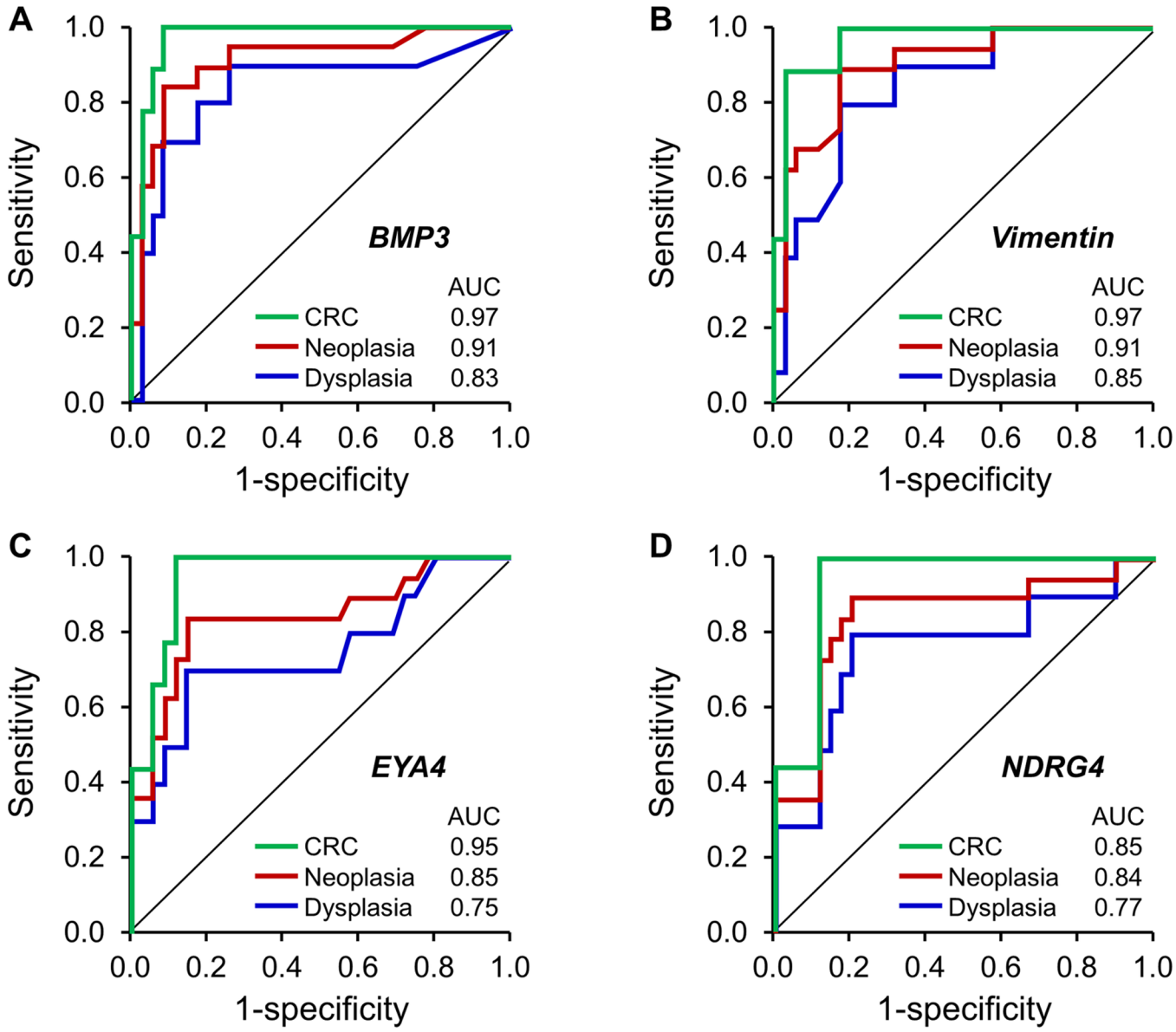

Figure 2.
Receiver Operating Characteristics Curve for Detection of Neoplasms by Stool Assay of Methylated Genes. Data plotted for A) *BMP3*, B) *Vimentin*, C) *EYA4*, and D) *NDRG4* gene markers. AUC = area under curve; CRC = colorectal cancer.

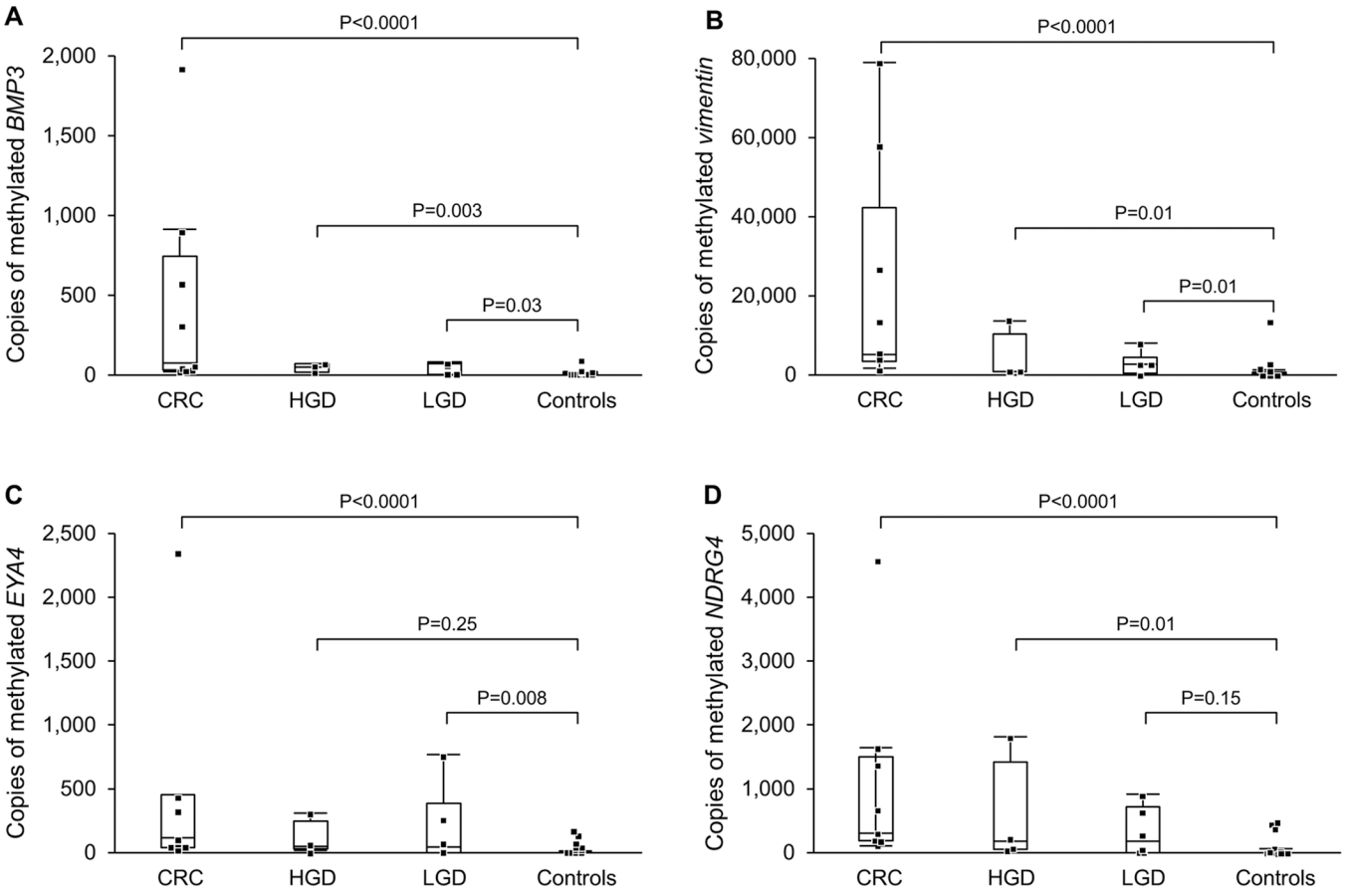

Figure 3.

Distributions of Methylated Gene Marker Levels in Stools from IBD Cases with Colorectal Neoplasia and from IBD Controls without Neoplasia. Data plotted for A) *BMP3*, B) *Vimentin*, C) *EYA4*, and D) *NDRG4* gene markers. CRC = colorectal cancer; HGD = high-grade dysplasia; LGD = low-grade dysplasia.

Table 1

Patient Characteristics for Tissue Study

	Cases N = 25	Controls N = 25
Male (%)	16 (64)	17 (68)
Mean age, years (SD)	52 (14.4)	50 (11.9)
Mean CUC duration, years (SD)	20.7 (9.2)	19.9 (8.3)
Extensive (%)	21 (84)	20 (80)
PSC (%)	4 (16)	3 (12)

SD, standard deviation
CUC, chronic ulcerative colitis
PSC, primary sclerosing cholangitis

Cases = Colorectal cancer in CUC, Controls = CUC without neoplasia

Table 2

Patient Characteristics for Stool Study

	Cases N = 19	Controls N = 35
CUC	17	25
Crohn's disease	2	10
% Male	63	63
Median age, years (range)	60 (45–72)	60 (45–77)
Median IBD duration, years (range)	30 (2–50)	14 (0–45) [1]
Extensive (%)[2]	17 (89)	19 (54)[3]
IBD Activity[4]		
Inactive (%)	8 (42)	12 (38)
Mild (%)	9 (45)	11 (34)
Moderate (%)	1 (5)	3 (9)
Severe (%)	1 (5)	6 (19)
PSC (%)	4 (21)	5 (16)

[1] p=0.0008

[2] Inflammation proximal to splenic flexure

[3] p=0.01

[4] Disease activity could not be confirmed for 3 control patients

CUC, chronic ulcerative colitis; IBD, inflammatory bowel disease; PSC, primary sclerosing cholangitis.

Cases = IBD with colorectal neoplasia, Controls = IBD without neoplasia

Table 3

IBD-Associated Colorectal Neoplasm Detection Rates by Stool Assay of Methylated DNA Markers

Specificity Cut-off, %	Sensitivity, % (95% CI)			
	mBMP3	*mVIM*	*mEYA4*	*mNDRG4*
CRC[1]				
94	89 (51–99)	89 (51–99)	66 (31–91)	44 (15–77)
91	100 (63–100)	89 (51–99)	78 (40–96)	44 (15–77)
89	100 (63–100)	89 (51–99)	100 (63–100)	100 (63–100)
Neoplasia[2]				
94	68 (43–86)	68 (43–86)	53 (29–74)	37 (17–61)
91	84 (60–96)	68 (43–86)	63 (39–82)	37 (17–61)
89	84 (60–96)	68 (43–86)	74 (48–90)	74 (48–90)
Dysplasia				
94	50 (20–80)	50 (20–80)	40 (14–73)	30 (8–65)
91	70 (35–91)	50 (20–80)	50 (20–80)	30 (8–65)
89	70 (35–91)	50 (20–80)	50 (20–80)	50 (20–80)

[1] CRC = colorectal cancer

[2] Neoplasia = CRC + premalignant dysplasia combined