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DNA methylation patterns in blood of patients with colorectal cancer and adenomatous colorectal polyps

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Colorectal cancer (CRC) screening rates are currently suboptimal. Blood-based screening could improve rates of earlier detection for CRC and adenomatous colorectal polyps. In this study, we evaluated the feasibility of plasma-based detection of early CRC and adenomatous polyps using array-mediated analysis methylation profiling of 56 genes implicated in carcinogenesis. Methylation of 56 genes in patients with Stages I and II CRC ($N = 30$) and those with adenomatous polyps ($N = 30$) were compared with individuals who underwent colonoscopy and were found to have neither adenomatous changes nor CRC. Composite biomarkers were developed for adenomatous polyps and CRC, and their sensitivity and specificity was estimated using five-fold cross validation. Six promoters (CYCD2, HIC1, PAX 5, RASSF1A, RB1 and SRBC) were selected for the biomarker, which differentiated CRC patients and controls with 84% sensitivity and 68% specificity. Three promoters (HIC1, MDG1 and RASSF1A) were selected for the biomarker, which differentiated patients with adenomatous polyps and controls with sensitivity of 55% and specificity of 65%. Methylation profiling of plasma DNA can detect early CRC with significant accuracy and shows promise as a methodology to develop biomarkers for CRC screening.

Introduction

In 2010, it is estimated that over 140,000 Americans will be diagnosed and over 48,000 will die from Colorectal Cancer (CRC).[1] Five year survival is highly dependent on tumor stage at time of detection. Among the unscreened, average risk US population the prevalence of CRC is 0.5–1%.[2] Diagnostic colonoscopy is the most commonly used modality for CRC screening though only a minority or Americans undergo it.[3] Colonoscopy quality is variable and depends on a number of factors including difficulty in detection of flat or depressed lesions, right-sided lesions, suboptimal bowel preparations and "interval" cancers with aggressive biology.[4–6]

Noninvasive screening methods for colorectal neoplasia should have high sensitivity for curable stage CRC as well as advanced precancerous lesions, affordability and specificity.[7] Currently commercially available stool tests of hemoccult-based testing (FOBT) and fecal immunochemical testing (FIT) do not detect the majority of advanced adenomas.[8] Lesion size, villous histology and multiplicity of polyps have been correlated with increased risk of future advanced colorectal neoplasia.[9]

Aberrant methylation of CpG islands at the promoter region of genes leads to transcriptional silencing of tumor suppressor genes.[10] Hypermethylation of CpG Islands in tumor suppressor genes has been reported for several cancers including CRC.[11,12] Methylation of tumor suppressor genes silenced by hypermethylation and detectable in the plasma or serum of patients with CRC has been shown to hold promise as a potential methodology for the detection of CRC.[13,14] Plasma DNA methylation profiles that assess methylation status at multiple sites have been used in biomarker development for detection in cell-free plasma DNA to reflect the primary disease.[15,16]

In this study, we examined whether patients with early CRC, (Stages I and II) and patients with adenomatous polyps could be differentiated from controls using DNA methylation profiling of cell-free circulating plasma. DNA Methylation was evaluated in 56 genes using MethDet56 platform in each sample. The most informative genes selected by a statistical algorithm as genes with the greatest difference in methylation between controls and patients with CRC and with adenomatous polyps respectively were included in the composite biomarker.[16,17]

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Table 1. Age (range), extent of dysplasia and tumor location in colorectal clinical specimens in enrolled patients

	Mean age	Dysplasia	Location of neoplasia
Controls ($N = 30$)	61.2 (40–80)	None	NA
Adenomatous Polyps ($N = 30$)	61.6 (40–84)	Advanced adenomas 17/30 (57%)	Right sided 7/30 (23%)
Colon Cancers ($N = 30$)	68.3 (49–85)	Adenocarcinoma; Stage I = 11; Stage II =19	Right Colon 8/30 (27%)

Material and Methods

Patient enrollment

The study was approved by the Institutional Review Boards of the Rush University Medical Center, Chicago, Illinois and University of Insubria, Italy. Controls were individuals evaluated and deemed medically appropriate for standard colorectal cancer screening by colonoscopy and had colonoscopy negative for either adenomatous changes or CRC. Patients could have cardiac, pulmonary, renal or liver disease but no chronic medical condition was represented in more than 10% of patients. Patients who had a history of a solid organ tumor were excluded. No cancer patients had neoadjuvant chemoradiotherapy. Colonoscopies in all patients were considered to have adequate preparation and were complete to the cecum. Colorectal cancers included patients with Stages I or II disease only. All CRC patients had surgical resections and no evidence of nodal disease. Sample Collection and DNA Isolation: Whole blood was obtained by venipuncture, collected in vacutainer tubes containing EDTA and stored at 4°C for a maximum of 2 hr. Blood was obtained within one week prior to the surgery in patients with CRC. For patients with polyps or controls blood was obtained on the day of colonoscopy. Tubes were centrifuged twice (2600*g*) for 10 min at 4°C to separate plasma from cellular elements. The plasma was then aliquoted and stored at −80°C. DNA was isolated using DNAzol BD and proteinase K as previously described and quantified by fluorimetry.[15,18]

Microarray-mediated methylation assay

The assay was done as previously described.[17,19,20,22] Briefly, DNA samples were split into two equal aliquots, and one was digested with *Hin6I* (Fermentas, Glen Burnie, MD) while the other was mock digested. Both samples were amplified *via* a multiplexed, nested polymerase chain reaction (PCR). 5-aminoallyl dUTP (Biotium, Hayward, CA) was added for the second PCR; products of the *Hin6I* digested DNA were labeled with Cy3, while products of the mock digested DNA were labeled with Cy5. Both labeled products were mixed and hybridized to custom printed DNA microarrays (Microarrays, Huntsville, AL). The slides were then washed and scanned using a Genepix 4000B Microarray Scanner (Molecular Devices, Union City, CA) and the data were analyzed using the Genepix Pro 6.0 software.

Statistical analysis

Methylation patterns in cfpDNA were determined using the MethDet 56 test in three different cohorts: CRC patients, colorectal adenoma patients and those without any adenomatous changes or CRC on colonoscopy. There was 85% power to detect an effect size of 0.8 in methylation ratio (difference divided by standard deviation) between any two groups.

Data were processed using a fixed cutoff approach as previously described.[15,17–19] First, the same PCR product from an undigested sample was divided into two parts, which were labeled with either Cy5 or Cy3 and used for hybridization with the microarray. This PCR product from an undigested DNA presented a model of a completely methylated fragment where the differences in fluorescent signal were dependent solely on the fluorescent dyes. The Cy5/Cy3 ratio ($r = 4$) for this DNA fragment was used as a threshold for binarization (methylated *vs.* unmethylated) of results. We next applied Fisher's exact test analysis with $p < 0.05$ to select the most differentially methylated genes. Finally, the most informative combination of genes was selected by naïve Bayes algorithm with 5-fold cross-validation to obtain sensitivity and specificity.

Results

Clinical specimens

The age of subjects and clinical characteristics are presented in Table 1. The control group free of adenomatous polyps, the adenomatous polyp group and the CRC group each contained 30 patients. Patients in all three groups were similar by age and gender. Eighteen of the patients with polyps had either villous histology or size $\geq$1 cm. The presence of right colon neoplasia was 7/30 (23%) in the polyp group and 8/30 (27%) in the CRC group. Among CRC patients 11/30 (37%) had Stage I disease and 19/30 (63%)—Stage II disease. Table 2 depicts the genes used in the MethDet 56 microarray.

Methylation data by the fixed cutoff approach to differentiate patients with CRC from healthy controls

Using the fixed cutoff approach, differentiation between patients with CRC *versus* controls (Fig. 1*a*) was more accurate than differentiation of patients with adenomatous polyps *versus* controls (Fig. 1*b*). Using the Fisher's Exact test between paired samples, seven genes were informative with a $p < 0.05$. The informative genes were (CYCD2, HIC, MDR1, PAX5, RASSF1A, RB1, SRBC). Next, naïve Bayes algorithm with 5-fold cross-validation was used to obtain the most informative panel of genes. This yielded a six gene panel (CYCD2, HIC1, PAX5, RASSF1A, RB1, SRBC). The sensitivity of this panel was 83.7% and the specificity was 67.9% to differentiate CRC patients from those without polyps

Table 2. The genes used in the MethDet 56 Microarray

ABCB1	*ACTB*	*APAF1*	*BRCA1*	*CALCA*	*CASP8*	*CCND2*	*CDH1*
CDKN1A	*CDKN1B*	*CDKN1C*	*CDKN2A*	*CDKN2B*	*DAPK1*	*DNAJC15*	*EDNRB*
EP300	*ESR1prA*	*ESR1prB*	*FABP3*	*FAS*	*FHIT*	*GPC3*	*GSTP*
HIC1	*HLTF*	*ICAM1*	*MCTS1*	*MGMT*	*MLH1*	*MSH2*	*MUC2*
MYOD1	*NR3C1*	*PAX5*	*PGK1*	*PGRdist*	*PGRprox*	*PLAU*	*PRDM2*
PRKCDBP	*PYCARD*	*RARB*	*RASSF1A*	*RB1*	*RPL15*	*S100A2*	*SCGB3A1*
SLC19A1	*SOCS1*	*SYK*	*TES*	*THBS1*	*TNSF11*	*TP73*	*VHL*

Negative controls include coding sequences of three genes (*ACTB*, *GADPH*, *TUBA3*) and heterologous DNA from Arabidopsis thaliana.

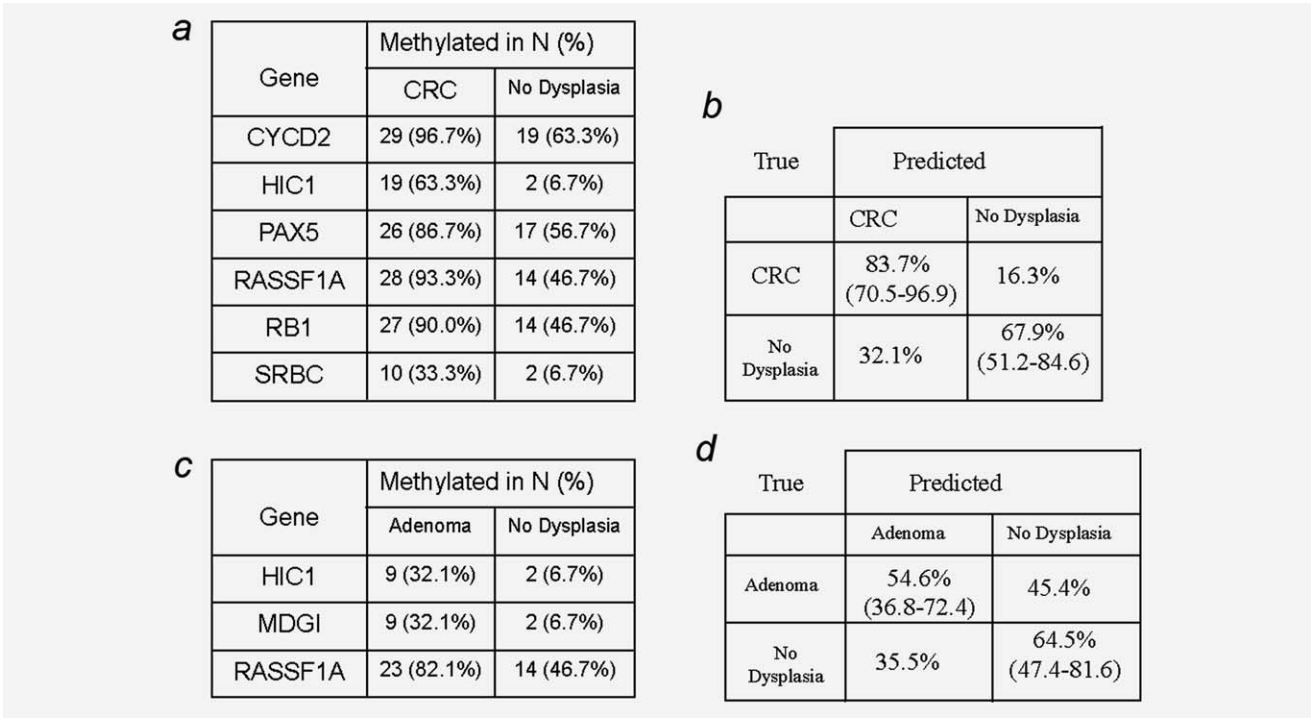

Figure 1. Analysis of results using the fixed cutoff approach following 5-fold cross validation. For each comparison the percentage of the sample that was methylated by binary fixed cut off approach is listed. (*a*) Individual informative genes that comprise the panel to differentiate patients with CRC from controls. (*b*) The informative genes panel yielded a sensitivity of 83.7% and specificity of 67.9% to differentiate CRC from controls. (*c*) Individual informative genes that comprise the panel to differentiate patients with adenoma from controls. (*d*) The informative genes as a panel yielded sensitivity of 54.6% and specificity of 64.5% to differentiate patients with adenomatous polyps from controls.

(Fig. 1*c*). In all comparisons, methylation status of informative genes was higher in CRC patients than in controls.

Methylation data by the fixed cutoff approach to differentiate patients with adenomatous Polyps from controls

Differentiation by the fixed cut off approach of patients with adenomatous polyps *versus* those without any polyps showed three informative genes by Fisher's Exact test with a $p < 0.05$ (HIC1, MDGI, RASSF1A). Naïve Bayesian analysis included all three genes (HIC1, MDG1, RASSF1A) which yielded a 54.6% sensitivity with a 64.5% specificity (Figs. 1*b* and 1*d*).

Table 3 lists the informative genes of the composite biomarker for CRC differentiation from controls (RASSF1A, HIC1, CYCD2, PAX5, RB1, SRBC) by the fixed cut-off approach. Using this binary assessment of methylation, all of the above six informative genes likewise had a higher frequency of methylation status in patients with adenomatous polyps than in healthy controls though all six genes were methylated in CRC patients. Two genes (HIC1; RASSF1A) were informative for differentiation of both adenomatous polyps and likewise CRC patients from controls though these cohorts were analyzed separately. This observation suggests that patients with adenomatous polyps and CRC have common genes that tend to be methylated at greater frequency than seen with healthy controls. The sensitivity and specificities of the six individual genes to differentiate controls without adenomatous lesions from CRC patients are also listed in Table 3.

Table 3. Informative (methylated) genes selected for composite biomarker for colorectal cancer and for colonic adenoma detection by the fixed cut-off approach

Gene	Informative for CRC	Informative for adenoma	Controls $N = 30$	Adenoma $N = 30$	Colorectal cancer $N = 30$
RASSF1A	+	+	14 (47%)	23 (82%)	28 (Se = 93%, Sp = 53%)
HIC1	+	+	2 (7%)	9 (30%)	19 (Se = 63%, Sp = 93%)
CYCD2	+	−	19 (63%)	23 (82%)	29 (Se = 97%,Sp = 37%)
PAX5	+	−	17 (57%)	21 (75%)	26 (Se = 87%, Sp = 43%)
RB1	+	−	14 (47%)	19 (68%)	27 (Se = 90%, Sp = 53%)
SRBC	+	−	2 (7%)	3 (11%)	10 (Se = 33%, Sp = 93%)

The number (and percentage) of samples with methylated gene is shown. Sensitivity (Se) and Specificity (Sp) for the informative genes that differentiated colorectal cancer from controls are listed.

Discussion

Mortality from CRC is reduced by early detection and removal of adenomatous polyps.[20,21,23,24] Currently commercially available stool tests of FOBT and FIT have shown some reduction in mortality in CRC.[20] Rates of screening for CRC are significantly lower than for other malignancies including breast (mammography), prostate (PSA) and cervix (pap smear) and most Americans do not undergo age appropriate screening for CRC by any methodology.[3,22] Failure to screen for CRC stems from multiple reasons including time, cost, privacy concerns as well as perceptions of procedural and bowel preparation related-pain and aversion to stool based noninvasive modalities. Sensitivity of the above mentioned noninvasive modalities is reduced in early stage cancers and adenomas.[23]

Promoter methylation of varied genes can differentiate patients with adenomatous polyps and CRC from controls with some accuracy in tissue and stool.[24–26] Plasma markers have been investigated for early detection of CRC and numerous individual genes including SEPT9 and ALX4 have been identified as potential biomarkers with limited sensitivity for detection of precancerous polyps.[27,28,32–34] Other individual promoters including TMEFF2, NGFR and MGMT, CDKN2A, RARB2, RASSF1A and APC have been suggested as potential biomarkers of CRC in plasma, but their accuracy remains limited.[29,30]

As methylation status of single genes have limited accuracy we sought to develop a methodology for the analysis of multiple different methylation sites simultaneously. This technology should allow identification of the most informative methylation sites or methylation profiling that can distinguish patients with colorectal malignant and premalignant lesions from controls to show promise as a potential screening modality. In this study we used the proof-of-principle platform for methylation analysis of 56 genes (MethDet-56) in order to compare methylation patterns in plasma from controls and patients with CRC and adenomatous polyps.

MethDet is a technique developed to produce a multigene methylation signature in each sample, so that selection of the most informative genes and their combinations becomes possible.[18] A set of six genes (CYCD2, HIC1, PAX5, RB1, SRBC; Fig. 3*c*) selected by a fixed cut-off approach provides a sensitivity of 84% and a specificity of 68% to discriminate CRC from healthy controls. Patients with adenomatous polyps could also be differentiated from healthy controls but to a lesser extent. This suggests that different and likely larger sets of genes are required to improve differential detection of patients with adenomatous polyp from controls.

HIC1 and RASSF1A, the two sites with preferential methylation in both the CRC and polyp plasma cohorts in this series have been described as informative genes for detection of CRC and polyps in tissue.[25,31,32] Increase in frequency of methylated RB1 in CRC has been described but is reported in plasma for the first time.[33] Preferential methylation of SRBC and PAX 5 in plasma of CRC patients is reported for the first time.[34,35] RASSF1A and PAX5 have been shown to be preferentially methylated in other solid organ malignancies. While patients with known malignancies in other organs were excluded, it is possible that occult malignancies may have impacted on our results.[36,37]

In this proof-of-principle study we demonstrate that patients with adenomatous polyps and CRC share common preferentially methylated sites (HIC1, RASSF1A) when compared separately to controls without adenomatous change. While this proof-of-principle study is not intended as a clinical-grade biomarker discovery, we have nonetheless identified genes—RASSF1A and HIC1—as preferentially methylated and informative in plasma in two separate cohorts (CRC and adenomatous polyps) compared with healthy controls. These common features suggest that differences in specific methylation sites may be present from lower grade dysplasia to malignant lesions. It is intriguing that the accuracy of detection of early CRC is higher than that of adenomatous polyps. This suggests that clinically higher grades of dysplasia might be detected better using this approach.

We intentionally sought after early cancers (Stages I and II) that would be of most benefit to detect by a screening modality. There are, however, several limitations to this proof-of-principle study, including homogeneity of the test population (only European Caucasian) and a lack of several genes that may have added to the accuracy of detection, *e.g.*, NDRG4, SEPT9, RUNX3 and ALX4.[28,29,38] The methylation patterns developed in this study should not be considered

actionable clinical-grade biomarkers. Nonetheless, they suggest that accurate biomarkers could be developed once larger, preferably genome-wide analysis of blood-based methylation profiles is completed.

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