Research Article

DNA Methylation of Phosphatase and Actin Regulator 3 Detects Colorectal Cancer in Stool and Complements FIT

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Abstract

Using a bioinformatics-based strategy, we set out to identify hypermethylated genes that could serve as biomarkers for early detection of colorectal cancer (CRC) in stool. In addition, the complementary value to a Fecal Immunochromostic Test (FIT) was evaluated. Candidate genes were selected by applying cluster alignment and computational analysis of promoter regions to microarray-expression data of colorectal adenomas and carcinomas. DNA methylation was measured by quantitative methylation-specific PCR on 34 normal colon mucosa, 71 advanced adenoma, and 64 CRC tissues. The performance as biomarker was tested in whole stool samples from in total 193 subjects, including 19 with advanced adenoma and 66 with CRC. For a large proportion of these series, methylation data for GATA4 and OSMR were available for comparison. The complementary value to FIT was measured in stool subsamples from 92 subjects including 44 with advanced adenoma or CRC. Phosphatase and Actin Regulator 3 (PHACTR3) was identified as a novel hypermethylated gene showing more than 70-fold increased DNA methylation levels in advanced neoplasia compared with normal colon mucosa. In a stool training set, PHACTR3 methylation showed a sensitivity of 55% (95% CI: 33–75) for CRC and a specificity of 95% (95% CI: 87–98). In a stool validation set, sensitivity reached 66% (95% CI: 50–79) for CRC and 32% (95% CI: 14–57) for advanced adenomas at a specificity of 100% (95% CI: 86–100). Adding PHACTR3 methylation to FIT increased sensitivity for CRC up to 15%. PHACTR3 is a new hypermethylated gene in CRC with a good performance in stool DNA testing and has complementary value to FIT. Cancer Prev Res; 5(3); 464–72. ©2011 AACR.

Introduction

Screening for colorectal cancer (CRC) is the most efficient strategy for reducing death from this devastating disease. Colonoscopy is the gold standard for the detection and removal of early lesions and is highly sensitive, but also invasive and costly (1, 2). For population-wide screening simple and noninvasive procedures like stool testing are preferred (3). In follow-up to the guaiac-based Faecal Occult Blood Test (FOBT), the more sensitive immunochemical fecal occult blood test (Fecal Immunochromostic Test or FIT; refs. 4–6) is now widely used in screening programs in Europe and Japan, and is expected to reduce CRC mortality by around 30% (7). This test performance though, still leaves room for improvement which could come from molecular stool tests like those testing for tumor DNA in stool. Multiple assays have been developed and evaluated for this purpose, but sensitivities still remain suboptimal (8–14). The recently introduced combination of mutation markers with DNA methylation markers has yielded substantially improved test performance (13, 15, 16). Methylation markers on their own, either alone or combined, have also yielded promising results, whereas the assays are technically less demanding (17–23). Methylation markers are appealing for CRC screening even more because DNA methylation is an early event in colorectal development, preceding chromosomal abnormalities, and mutations (24).

Yet, the ultimate marker, or combinations of markers, for stool DNA testing still remains to be determined. To this end, in this study, we aimed to identify new hypermethylated genes in CRC by applying dedicated bioinformatics to microarray expression data of colorectal adenomas and carcinomas and to explore their potential in whole stool
DNA testing for CRC (25, 26). Furthermore, we set out to evaluate complementary value of such markers to FIT in a series of stool subsamples.

Materials and Methods

Cell line authentication

The CRC cell line HT29 was obtained from the American Type Culture Collection (ATCC; GlaxoSmithKline). COLO205, Colo320, HCT 116, and RKO cell lines were kindly provided by Dr. G.J. Peters, Department of Oncology, VU University Medical Center, Amsterdam, the Netherlands. The method for authentication was by array comparative genomic hybridization (aCGH, 244 k Agilent oligonucleotide platform), conducted at the VU University Medical Center, Amsterdam, the Netherlands, most recently in October 2008. The patterns of chromosomal changes observed were in concordance to the previously described chromosomal changes in these cell lines (27). LS513 was kindly provided by Dr. F. Praz, Centre de Recherche Saint-Antoine, Paris, France. Array CGH confirmed the genomic profile as described in literature (the Wellcome Trust Sanger Institute Cancer Genome Project web site; ref. 28).

Strategy for methylation marker selection

The strategy used for identification and validation of new colon cancer-specific methylation markers included both bioinformatics analysis of microarray-based mRNA expression data and experimental validations of methylation levels as outlined in Supplementary Fig. S1. First, genes downregulated in CRC compared with adenomas were subjected to a bioinformatics strategy for predicting cancer-specific methylation (25). In the experimental validation, the presence of DNA methylation was tested by methylation-specific PCR (MSP) using the BioTrove OpenArray platform (Ref. 29; BioTrove, Inc.), and high-throughput LightCycler assays. Full details are provided in the Supplementary Methods.

Strategy for evaluating PHACTR3 methylation as a marker in CRC tissue and stool

Cell lines. For validating PHACTR3 methylation in CRC 2 different regions around the Transcription Start Site (TSS) were investigated for the presence of methylation in 6 CRC cell lines; regions –149 to –63 bp and +411 to +526 relative to the TSS. Next, HT29 and HCT116 cells were treated with 5-aza-2’-deoxycytidine (5-AZA) to evaluate the effect of demethylation on mRNA expression.

Tissue. PHACTR3 methylation levels in the region +411 to +526 relative to the TSS and mRNA expression were measured in cancer and matched normal tissue samples from 9 CRC patients by quantitative MSP and quantitative RT-PCR, respectively. Then, PHACTR3 methylation levels were evaluated in an independent series of 34 normal colon mucosa tissue samples from cancer-free patients, 71 advanced adenomas, and 64 carcinomas. Finally, to evaluate the specificity of PHACTR3 hypermethylation for CRC, methylation levels in other tumor types were analyzed, being tumor tissue samples from 44 breast, 20 cervix, 20 lung, 11 esophagus (5 adenocarcinomas and 6 squamous cell carcinomas), 20 gliomas, 19 pancreas, and 15 stomach.

Stool. To test the performance of PHACTR3 in a stool-based methylation test for CRC, a collection of 193 whole stool samples was split in a training set and a validation set of equal sizes. The training set consisted of a total of 100 stool samples, of which 66 from patients without colorectal neoplasia (58 healthy controls, 4 patients with colonic diverticula and 4 patients with hemorrhoids), 9 from patients with hyperplastic polyps, 3 from patients with nonadvanced adenoma, and 22 from CRC patients. The training set was designed with more controls then cases, to better assess specificity. The validation set consisted of a total of 93 stool samples, of which 30 from healthy individuals, 19 from patients with advanced adenoma and 44 from CRC patients. The validation set contained more advanced neoplasia, including advanced adenomas, to better assess sensitivity. (Patient characteristics are described in Supplementary Table S3). In addition, methylation data for 2 other markers, that is, GATA4 and OSMR were available for comparison in 95% of cases and 91% of controls from the training set and in all stool samples from the validation set tested for PHACTR3 (18, 20).

Moreover, to examine the complementary value to FIT, an independent series of 92 stool subsamples was analyzed for both FIT and PHACTR3 methylation. This stool series originated from a retrospective collection from referral subjects, and included 48 stool samples from subjects without colon neoplasia, 24 from patients with advanced adenomas and 20 from patients with carcinomas. All details on tissue and stool collection, sample processing, and methodologies used, are presented in the Supplementary Methods.

Statistical analysis of DNA methylation in tissues and stool

Mean differences in methylation or mRNA expression levels in tissue samples were analyzed with the Mann–Whitney test or ANOVA. The relation between methylation levels in stool and the presence or absence of an advanced lesion was studied by receiver operator characteristic (ROC) analysis. The Area under the curve (AUC) was used as a measure of the test performance. CIs of proportions were calculated using the Wilson score method. To test whether age or gender were confounders in the relation between methylation levels and the presence or absence of a lesion, linear regression was used. For the combination of FIT and PHACTR3 methylation, we used a distribution-free rank-based method (30) to calculate linear combination of the 2 markers giving highest diagnostic accuracy. Sensitivities were compared with FIT or PHACTR3 methylation alone at fixed specificities of 92%, 96%, and 98% using McNe mar’s test. Calculations for the combination of FIT and PHACTR3 methylation was carried out in the R package (version 2.8.1.). All other analyses were carried out using
SPSS software (version 15.0; SPSS Inc.). Values of $P \leq 0.05$ were considered statistically significant.

Results

Bioinformatics for discovery of candidate genes

A total of 397 genes were downregulated in carcinomas compared with adenomas as determined by mRNA expression microarray analysis (Wilcoxon rank test $P < 0.05$; $\text{FDR} < 0.05$; ref. 26). In 284 of these genes, a reported transcription start site (TSS) could be identified. These were subsequently subjected to a bioinformatics approach to predict cancer-specific methylation (25). This yielded 18 candidate genes, of which Phosphatase and actin regulator 3 (PHACTR3, NM_080672) was the only one that passed all further steps of experimental validation (see Supplementary Fig. S1 and Supplementary Table S1).

PHACTR3 methylation and mRNA expression analysis in CRC cell lines and cancer and matched normal tissues

PHACTR3 harbors a dense CpG island located $-678$ and $+1,353$ bp relative to the TSS (GC content: 65%, $\text{CpG(obs)/CpG(exp)}$: 1.17). We designed conventional MSP primers in a region $-149$ to $-63$ bp and $+411$ to $+526$ relative to the TSS (see Supplementary Methods and Supplementary Fig. S2). The region $+411$ to $+526$ relative to the TSS is located in exon 1 and is the same region as analyzed at the initial screen and validation of methylation status (see Supplementary Fig. S1 and Supplementary Fig. S2A). Of 6 cell lines tested, only HCT116 showed methylation in the region $-149$ to $-63$ bp relative to the TSS. For the region $+411$ to $+526$ relative to the TSS, methylation was found in all 6 cell lines tested (see Supplementary Fig. S2). At the mRNA level these 6 CRC cell lines only showed marginal PHACTR3 expression levels compared with the positive control (brain tissue; see Supplementary Fig. S2). Treatment of HT29 and HCT116 with the demethylating agent 5-AZA resulted in reexpression of the gene (see Supplementary Fig. S2), consistent with PHACTR3 expression being downregulated by methylation in these cell lines.

Next, PHACTR3 methylation levels and mRNA expression were measured in cancer tissue and matched normal tissue from 9 CRC patients. All 9 CRC tissue samples showed significantly increased methylation levels compared with their normal counterparts. Although mRNA expression levels were low in both normal and tumor tissues, still in 6 of 9 tumors PHACTR3 was significantly downregulated compared with expression levels in their normal counterparts (see Fig. 1).

PHACTR3 methylation analysis in colorectal advanced adenoma and carcinoma tissues

To confirm the differential levels of PHACTR3 methylation in CRC compared with normal mucosa, we tested an independent series of tissue samples and included a set of advanced adenomas as well. PHACTR3 methylation levels were 72-fold and 71-fold higher in advanced adenoma and carcinoma samples, respectively, compared with normal mucosal samples (see Fig. 2A, $P < 0.01$). ROC analysis yielded an AUIC of 0.93 (95% CI: 0.87–0.98) for CRC and 0.95 (95% CI: 0.93–0.98, see Fig. 3A) for advanced neoplasia (advanced adenomas and carcinomas; see Fig. 3). When fixing the cutoff for specificity at 100%, 72% of CRC and 81% of advanced neoplasia could be discriminated from normal mucosa. No significant differences in methylation levels were observed between advanced adenoma and carcinoma tissue samples or between carcinomas of different UICC stages ($P = 0.5$ and $P = 0.07$, respectively). Age or gender was no confounding factors ($P = 0.1$ and $P = 0.9$, respectively). Methylation levels in other tumor types showed high levels of methylation in tissue samples from tumors of the intestinal tract (pancreatic, gastric, and esophageal cancer) and in cervical cancer, whereas lower levels of methylation were seen in cancers of lung and bladder, and little or no methylation was seen in cancers of breast, prostate, and brain (glioma; Supplementary Fig. S3).

Sensitivity and specificity of PHACTR3 methylation in stool for detecting colorectal cancer

To investigate the performance of PHACTR3 methylation as a biomarker for CRC detection in stool, we measured...
methylation levels in 2 independent series of stool-derived DNA samples (see Fig. 2B and C). A training set was used to determine the optimal cutoff to detect CRC patients compared with controls and nonadvanced adenoma patients. The validation set was used to validate the results from the training set and to test the detection rate of advanced adenomas.

Training set. The training set consisted of 100 stool samples from individuals who all had undergone complete colonoscopy. ROC analysis of CRC (n = 22) compared with nonadvanced adenomas and control samples (n = 78) yielded an AUC of 0.77 (95% CI: 0.64–0.90, see Fig. 3B). Maximum sensitivity at a fixed specificity of 95% (95% CI: 87–98) was reached at a cutoff value of 82.5 relative copies. At that cutoff, sensitivity was 55% (95% CI:33–75) for detecting CRC.

Validation set. The validation set consisted of 93 stool samples from individuals who all had undergone complete colonoscopy. ROC analysis with advanced neoplasia (44 CRC and 19 advanced adenoma) compared with healthy controls (n = 30) resulted in an AUC of 0.83 (95% CI: 0.75–0.91, see Fig. 3C). Using a cutoff of value of 82.5 relative copies as defined with the training set yielded a specificity of 100% (95% CI: 86–100), a sensitivity of 66% (95% CI: 50–79) to detect CRC, and a sensitivity of 32% (95% CI: 14–57) to detect advanced adenomas. Using a cutoff of 28 relative copies, the highest possible sensitivity for advanced adenomas was 53% (95% CI: 32–73), at a specificity of 93% (95% CI: 79–98). Age or gender was no confounding factor (P = 1.0 and P = 0.4, respectively).

Performance of PHACTR3 in comparison with other stool methylation markers
To compare the test performance of PHACTR3 methylation to other stool methylation markers, detection rates were compared with those obtained with the previously published markers, GATA4 (18) and OSMR (20), which were tested by QMSP in a large proportion of the current training and validation series of stool samples. With the stool training set, the cutoffs for GATA4 and OSMR were determined to detect CRC at equal specificities as PHACTR3. Table 1 shows the AUC, the cutoffs, sensitivities and specificities of these 3 markers. Compared with OSMR, in the training set, PHACTR3 showed a higher AUC and sensitivity (52% vs. 29%) for carcinomas, and in the validation set PHACTR3 showed a higher sensitivity for both advanced adenomas (32% vs. 21%) and carcinomas (66% vs. 43%) with higher specificity (100% vs. 90%).
PHACTR3 and GATA4 showed equal AUCs and comparable sensitivities (52% vs. 57%) for detecting carcinomas in the training set. In the validation set, PHACTR3 showed a higher sensitivity for both advanced adenomas (32% vs. 16%) and carcinomas (66% vs. 39%) than GATA4, with higher specificity (100% vs. 93%).

The combination of these 3 markers, calling positive when at least 1 of the 3 markers would be positive, increased the sensitivity to detect advanced adenoma from 32% to 42% and the sensitivity to detect carcinoma from 52% to 62% (training set) and from 66% to 68% (validation set), however, at cost of specificity (decreased from 94% to 87% and from 100% to 83% for the training and validation set, respectively).

Combination of FIT and PHACTR3 methylation in stool
To investigate whether FIT and PHACTR3 methylation would have complementary value for detecting colorectal
cancer and advanced neoplasia, both tests were evaluated in an independent series of stool subsamples. Because for DNA methylation analysis, the stool subsamples were processed in a different way compared with whole stool samples, different cutoffs were used, based on ROC analysis, to determine sensitivity and specificity (see Supplementary Methods). The sensitivities to detect advanced adenomas were 21% (5/24, 95% CI: 9–40) for PHACTR3 and 21% (5/24, 95% CI: 9–40) for FIT. Combining PHACTR3 with FIT, meaning positive for at least 1 of the 2 measurements, increased the sensitivity to 33% (7/24, 95% CI: 18–53). The sensitivities to detect CRC were 50% (10/20, 95% CI: 30–70) for PHACTR3 and 65% (13/20, 95% CI: 43–82) for FIT. Combining PHACTR3 with FIT increased the sensitivity to 95% (19/20, 95% CI: 76–99). The specificity of the combination remained high [94% (45/48, 95% CI: 83–98) compared with 96% (47/48, 95% CI: 86–99) for PHACTR3 alone and 98% (47/48, 95% CI: 89–100) for FIT alone]. In addition, a positive test for both FIT and PHACTR3, occurring in 7 of the 44 advanced neoplasia cases (see Supplementary Fig. S4), revealed 100% specificity.

To evaluate whether the sensitivity of the combination of FIT and PHACTR3 was significantly higher than the sensitivity of either FIT or PHACTR3 methylation alone, we compared the sensitivities at equal specificity of 92%, 96%, and 98% (see Supplementary Methods). ROC analysis resulted in an AUC of 0.97 (95% CI: 0.93–1.0) for CRC and 0.79 (95% CI: 0.69–0.92) for advanced neoplasia (see Fig. 4), with sensitivity/specificity combinations of 61%/92%, 55%/96%, and 48%/98% (see Table 2 for advanced adenoma and cancer separately). At these specificities, sensitivities for detecting CRC increased up to 15%

Table 1. Test performances of PHACTR3, GATA4, and OSMR

<table>
<thead>
<tr>
<th></th>
<th>PHACTR3</th>
<th>GATA4</th>
<th>OSMR</th>
</tr>
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<tbody>
<tr>
<td><strong>Training set</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AUC (95% CI)</td>
<td>0.76 (0.62–0.89)</td>
<td>0.76 (0.62–0.90)</td>
<td>0.60 (0.45–0.75)</td>
</tr>
<tr>
<td>Cutoff</td>
<td>82.5</td>
<td>16.5</td>
<td>3.6</td>
</tr>
<tr>
<td>Sensitivity CRC (n = 21)</td>
<td>52%</td>
<td>57%</td>
<td>29%</td>
</tr>
<tr>
<td>Specificity (n = 71)</td>
<td>94%</td>
<td>94%</td>
<td>94%</td>
</tr>
<tr>
<td><strong>Validation set</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cutoff</td>
<td>82.5</td>
<td>16.5</td>
<td>3.6</td>
</tr>
<tr>
<td>Sensitivity AA (n = 19)</td>
<td>32%</td>
<td>16%</td>
<td>21%</td>
</tr>
<tr>
<td>Sensitivity CRC (n = 44)</td>
<td>66%</td>
<td>39%</td>
<td>43%</td>
</tr>
<tr>
<td>Specificity (n = 30)</td>
<td>100%</td>
<td>93%</td>
<td>90%</td>
</tr>
</tbody>
</table>

Abbreviations: AA, Advanced adenoma; GATA4, GATA-binding protein 4; OSMR, oncostatin M receptor.
using the combination of FIT and PHACTR3 methylation compared with FIT alone, although statistical significance was not reached.

Discussion

Stool-based DNA testing is an appealing approach for noninvasive early detection of CRC. Both DNA mutations and DNA promoter hypermethylation have been investigated as targets of potential screening assays, and especially panels of markers have shown promising performance in initial studies. In this study, we applied a bioinformatics approach for identifying novel hypermethylated genes in CRC. This resulted in the identification of PHACTR3 as a new hypermethylated gene in CRC, which showed to have complementary diagnostic power to FIT in a pilot series.

PHACTR3 was first described in 2003 as a protein associated with the nuclear scaffold in human promyelocytic HL-60 leukemia cells. It was found to bind to actin and to the catalytic subunit of Protein Phosphatase 1 (PP1), which in turn can trigger apoptosis and can inhibit oncogenic signaling due to inhibition of the function of PP1 (31). Induced expression of PHACTR3 was first described in 2003 as a protein associated with the nuclear scaffold in human promyelocytic HL-60 leukemia cells. It was found to bind to actin and to the catalytic subunit of Protein Phosphatase 1 (PP1), which in turn can trigger apoptosis and can inhibit oncogenic signaling due to inhibition of the function of PP1 (32). Induced expression of PHACTR3 in HeLa cervical cancer cells stimulated cell spreading and motility (33). Finally, in a small study of non–small cell lung cancer patients gene mutations were found in 6/20 patients, which was associated with shortened overall survival (34). Yet, looking at PHACTR3 mRNA expression, abundant expression has been found in adult normal human brain and to a lesser extent in ovary, but not in other organs (31). Accordingly, in this study, we observed high PHACTR3 mRNA expression in human brain tissue, but much lower expression levels in normal colon tissues and again even lower expression in 6 of 9 matched tumor tissues. Although PHACTR3 showed high methylation levels in these 6 tumor tissue samples, methylation levels were equally high in the 3 tumor tissues from patients in which mRNA expression was not decreased compared with their normal counterparts. In addition, a direct correlation between the level of methylation and level of mRNA expression in all 9 tumor tissues could not be shown (Pearson correlation of \( -0.3, P = 0.5 \), data not shown). Treatment of HT29 and HCT116 cells with the demethylating agent 5-aza did result in reexpression of PHACTR3, however, consistent with the recent observation that methylation in the region of the first exon, which is the case for PHACTR3, is tightly linked to transcriptional silencing (35). Yet, the possibility that the observed reexpression of PHACTR3 could be secondary to demethylation of another gene or locus then PHACTR3 itself cannot be excluded. Although a direct relationship between PHACTR3 hypermethylation and silencing of expression in CRC remains to be established, the fact that PHACTR3 methylation is highly associated with cancer still makes it an interesting candidate biomarker.

Levels of PHACTR3 methylation in CRC tissues were prominently high and could significantly discriminate advanced adenoma and carcinoma from normal mucosa at the tissue level. High methylation levels are important for obtaining a good signal to noise ratio in a stool-based assay, especially when lesions to be detected are small. Interestingly, advanced adenoma tissue samples showed methylation levels as high as carcinoma tissues, making PHACTR3 methylation attractive as a biomarker, in compliance with recent guidelines which have stated that the detection of advanced adenomas and not only early carcinoma should be the goal of CRC screening (36).

When further exploring its potential as a biomarker, in whole stool samples a sensitivity of 55% to 66% for detecting CRC and a sensitivity of 32% for detecting advanced adenoma was observed at a specificity of 95% to 100%. The test performance of PHACTR3 can be further improved, which is illustrated by the higher AUC in tissues compared with stool, in particular the sensitivity to detect advanced adenomas. With the currently used method

| Abbreviation: AA, Advanced adenoma. |

### Table 2. Cutoffs and sensitivities of FIT, PHACTR3 methylation, and their combination (Y) at equal specificities

<table>
<thead>
<tr>
<th>Specitivity</th>
<th>Cutoff</th>
<th>Sensitivity</th>
<th>Cutoff</th>
<th>Sensitivity</th>
<th>P</th>
<th>Cutoff</th>
<th>Sensitivity</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>AA</td>
<td>92%</td>
<td>1.0</td>
<td>33%</td>
<td>0.2</td>
<td>29%</td>
<td>1.0</td>
<td>16.0</td>
<td>33%</td>
</tr>
<tr>
<td>CRC</td>
<td>92%</td>
<td>1.0</td>
<td>90%</td>
<td>0.2</td>
<td>60%</td>
<td>0.2</td>
<td>19.5</td>
<td>95%</td>
</tr>
<tr>
<td>Advanced neoplasia</td>
<td>92%</td>
<td>1.0</td>
<td>59%</td>
<td>0.2</td>
<td>43%</td>
<td>0.2</td>
<td>16.0</td>
<td>61%</td>
</tr>
<tr>
<td>AA</td>
<td>96%</td>
<td>49.5</td>
<td>25%</td>
<td>0.7</td>
<td>17%</td>
<td>0.7</td>
<td>55.5</td>
<td>25%</td>
</tr>
<tr>
<td>CRC</td>
<td>96%</td>
<td>30.5</td>
<td>75%</td>
<td>0.7</td>
<td>50%</td>
<td>0.3</td>
<td>38.0</td>
<td>90%</td>
</tr>
<tr>
<td>Advanced neoplasia</td>
<td>96%</td>
<td>30.5</td>
<td>48%</td>
<td>0.7</td>
<td>32%</td>
<td>0.2</td>
<td>38.0</td>
<td>55%</td>
</tr>
<tr>
<td>AA</td>
<td>98%</td>
<td>99.5</td>
<td>21%</td>
<td>1.5</td>
<td>17%</td>
<td>1.0</td>
<td>81.5</td>
<td>25%</td>
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<tr>
<td>CRC</td>
<td>98%</td>
<td>86.5</td>
<td>65%</td>
<td>1.1</td>
<td>50%</td>
<td>0.6</td>
<td>78.0</td>
<td>75%</td>
</tr>
<tr>
<td>Advanced neoplasia</td>
<td>98%</td>
<td>86.5</td>
<td>41%</td>
<td>1.1</td>
<td>32%</td>
<td>0.5</td>
<td>78.0</td>
<td>48%</td>
</tr>
</tbody>
</table>

NOTE: P values are based on the comparison with FIT.
higher sensitivities could also be reached, however, at cost of specificity. The highest sensitivity that could be reached to detect advanced adenomas for example was 53%, resulting in a specificity of 93%. With these test performances, PHACTR3 methylation is one of the best performing single methylation markers described so far [14]. Also in comparison to 2 previously published stool methylation markers GATA4 and OSMR, PHACTR3 showed superior test performance.

A strategy to improve test performance is to combine multiple markers in a single assay. Especially the high specificity makes PHACTR3 attractive as a marker for such a panel that would have increased sensitivity without major effects on specificity. The combination of PHACTR3 with GATA4 and OSMR in this study indeed did increase sensitivity, but at cost of specificity, which mainly is due to the lower individual specificities of GATA4 and OSMR. Nevertheless, it will be difficult to reach 100% sensitivity with methylation markers only, because a portion of CRCs have no or low frequencies of methylated genes, the so-called CpG Island Methylator Phenotype (CIMP)-negative tumors [37]. Therefore, an attractive alternative could be to combine methylation markers with a completely different marker like FIT. Because FIT is already being used in several screening programs throughout the world and logistics have already been put in place, adding a DNA methylation marker would be relatively easy. We therefore carried out a pilot study on the performance of PHACTR3 methylation in combination with FIT. The combination of FIT and PHACTR3 methylation increased the sensitivity for advanced neoplasia, that is, adenomas and CRC taken together, while maintaining a high specificity. These experiments were done in a series of stool subsamples, which would be a good alternative for whole stool samples concerning logistics, stool processing, and storage of samples in large-scale screening programs. Because these stool subsamples were processed in a different manner compared with the whole stool samples described above, systematic differences exist when comparing these results to those from whole stool samples (4). Still, the results obtained show the power of combining FIT with a DNA methylation marker. This is in line with recent findings that showed an improved test performance of combining other molecular markers (i.e., APC, BAT26, and long-DNA) with FOBT [12, 38].

In conclusion, using a bioinformatics approach, PHACTR3 was identified as a new hypermethylated gene in CRC. Although we could not unravel the functional effect of PHACTR3 hypermethylation in CRC, we clearly showed its high potential as a biomarker in stool-based DNA testing. Furthermore, this study suggests that combining PHACTR3 methylation with FIT could be particularly promising. The full potential of this marker or its combination with FIT awaits validation in a larger, well-controlled cohort study to test its performance in an asymptomatic population.

Disclosure of Potential Conflict of Interest
J. Louwage and W. van Criekinge were employees of MDxHealth and are holding stock options. G.A. Meijer is a consultant to MDxHealth and receives research funding.

Acknowledgments
The authors thank Iedan Verly and Kim Wouters for their technical assistance.

Grant Support
This study was financially supported by SenterNovum (ISO52034), ZonMW (61200022), and performed within the framework of CITEM, the Center for Translational Molecular Medicine, DeCoDe project (grant 03O-101).

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Received June 20, 2011; revised November 15, 2011; accepted November 17, 2011; published OnlineFirst December 1, 2011.

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Colorectal Cancer in Stool and Complements FITDNA Methylation of Phosphatase and Actin Regulator 3 Detects

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