DNA methylation of phosphatase and actin regulator 3 detects colorectal cancer in stool and complements FIT

Linda JW Bosch¹, Frank A Oort², Maarten Neerincx², Carolina AJ Khalid-de Bakker³, Jochim S Terhaar sive Droste², Veerle Melotte⁴, Daisy MAE Jonkers³, Ad AM Masclee³, Sandra Mongera¹, Madeleine Grooteclaes⁴, Joost Louwagie⁴, Wim van Criekinge⁴, Veerle MH Coupé⁵, Chris JJ Mulder², Manon van Engeland⁶, Beatriz Carvalho¹, Gerrit A Meijer¹

Affiliations of authors: ¹Department of Pathology, ²Gastroenterology and Hepatology and ⁵Clinical Epidemiology and Biostatistics, VU University Medical Center, Amsterdam, The Netherlands; ³Department of Internal Medicine, Division of Gastroenterology & Hepatology and ⁶Department of Pathology, GROW - School for Oncology and Developmental Biology, Maastricht University Medical Center, Maastricht, The Netherlands; ⁴MDxHealth, SA, Liège, Belgium

Running title: PHACTR3 methylation as stool DNA test

Key-words: PHACTR3, FIT; stool DNA test; biomarker; CRC; screening

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Correspondence: Gerrit A Meijer, MD, PhD, Dept. of Pathology, VU University Medical Center, PO Box 7057, 1007 MB, Amsterdam, The Netherlands; phone: +31204444772; fax number: +31204442964; e-mail: ga.meijer@vumc.nl
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 Immunochemical 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 sub-samples from 92 subjects including 44 with advanced adenoma or CRC.

Phosphatase and Actin Regulator 3 (PHACTR3) was identified as a novel hypermethylated gene showing >70-fold increased DNA methylation levels in advanced neoplasia compared to normal colon mucosa. In a stool training set, PHACTR3 methylation showed a sensitivity of 55% (95% confidence interval [CI] 33-75%) for CRC and a specificity of 95% (CI 95% 87-98%). In a stool validation set, sensitivity reached 66% (CI 95% 50-79%) for CRC and 32% (CI 95% 14-57%) for advanced adenomas at a specificity of 100% (CI 95% 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.
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 a simple and non-invasive 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 Immunochemical Test or FIT)(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, while the assays are technically less demanding(17-23). Methylation markers are appealing for CRC screening even more since 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 the present 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 sub-samples.

Materials & Methods

Cell line authentication

The CRC cell line HT29 was obtained from the American Type Culture Collection (LGC Standards GmbH, Wesel, Germany). 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, 244k Agilent oligonucleotide platform), performed 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(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 figure 1. First, genes downregulated in CRC compared to 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(29) (BioTrove, Inc. Woburn, MA, USA), 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 two different regions around the Transcription Start Site (TSS) were investigated for the presence of methylation in six 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 as well as mRNA expression were measured in cancer and matched normal tissue samples from nine 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 non-advanced adenoma, and 22 from CRC patients. The training set was designed with more controls than cases, to better assess specificity. The validation set consisted of a total of 93 stool samples, 30 of which 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 3). In addition, methylation data for two other markers, i.e. 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 sub-samples 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. Confidence intervals 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 two markers giving highest diagnostic accuracy. Sensitivities were compared to FIT or PHACTR3 methylation alone at fixed specificities of 92%, 96%, and 98% using McNemar’s test. Calculations for the combination of FIT and PHACTR3 methylation was performed in the R package (version 2.8.1.). All other analyses were performed using SPSS software (version 15.0; SPSS Inc., Chicago, IL, USA). P-values ≤0.05 were considered statistically significant.

Results

Bioinformatics for discovery of candidate genes

A total of 397 genes were downregulated in carcinomas compared to adenomas as determined by mRNA expression microarray analysis (Wilcoxon Rank test p-value <1e-5 and Thas p-value <0.05 (FDR < 0.05))(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 figure 1 and supplementary table 1).

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 +1353 bp relative to the TSS (GC content: 65 %, 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 figure 2). 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 figure 1 and supplementary figure 2A). Of six 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 six cell lines tested (see supplementary figure 2). At the mRNA level these six CRC cell lines only showed marginal PHACTR3 expression levels compared to the positive control (brain tissue) (see
supplementary figure 2). Treatment of HT29 and HCT116 with the demethylating agent 5-AZA resulted in re-expression of the gene (see supplementary figure 2), consistent with PHACTR3 expression being downregulated by methylation in these cell lines.

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

**PHACTR3 methylation analysis in colorectal advanced adenoma and carcinoma tissues**

To confirm the differential levels of PHACTR3 methylation in CRC compared to 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 to normal mucosa samples (see figure 2A, \( p < 0.01 \)). ROC analysis yielded an AUC of 0.93 (CI\(_{95\%} = 0.87-0.98\)) for CRC and 0.95 (CI\(_{95\%} = 0.93-0.98\), see figure 3A) for advanced neoplasia (advanced adenomas and carcinomas) (see figure 3). When fixing the cut-off 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 nor between carcinomas of different UICC stages (\( p = 0.5 \) and \( p=0.07 \), respectively). Age or gender were 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) as well as in cervical cancer, while 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 figure 3).

**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 two independent series of stool derived DNA samples (see figure 2B and 2C). A training set was used to determine the optimal cut-off to detect CRC patients compared
to controls and non-advanced 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 to non-advanced adenomas and control samples (n=78) yielded an AUC of 0.77 (CI95% = 0.64-0.90, see figure 3B). Maximum sensitivity at a fixed specificity of 95% (95% CI = 87-98%) was reached at a cut off value of 82.5 relative copies. At that cut off, 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 to healthy controls (n=30) resulted in an AUC of 0.83 (CI95% = 0.75-0.91, see figure 3C). Using a cut off of value of 82.5 relative copies as defined with the training set yielded a specificity of 100% (CI95% = 86-100%), a sensitivity of 66% (CI95% = 50-79%) to detect CRC, and a sensitivity of 32% (CI95% = 14-57%) to detect advanced adenomas. Using a cut off of 28 relative copies, the highest possible sensitivity for advanced adenomas was 53% (CI95% = 32-73%), at a specificity of 93% (CI95% = 79-98%). Age or gender were no confounding factors (p=1.0 and p=0.4, respectively).

**Performance of PHACTR3 in comparison to other stool methylation markers**
In order to compare the test performance of PHACTR3 methylation to other stool methylation markers, detection rates were compared to 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 cut-offs for GATA4 and OSMR were determined to detect CRC at equal specificities as PHACTR3. Table 1 shows the AUC, the cut-offs, sensitivities and specificities of these three markers. Compared to OSMR, in the training set, PHACTR3 showed a higher AUC and sensitivity (52% versus 29%) for carcinomas, and in the validation set PHACTR3 showed a higher sensitivity for both advanced adenomas (32% versus 21%) and carcinomas (66% versus 43%) with higher specificity (100% versus 90%). PHACTR3 and GATA4
PHACTR3 methylation as stool DNA test

showed equal AUCs and comparable sensitivities (52% versus 57%) for detecting carcinomas in the training set. In the validation set, PHACTR3 showed a higher sensitivity for both advanced adenomas (32% versus 16%) and carcinomas (66% versus 39%) than GATA4, with higher specificity (100% versus 93%).

The combination of these three markers, calling positive when at least one of the three 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 sub-samples. Because for DNA methylation analysis, the stool sub-samples were processed in a different way compared to whole stool samples, different cut-offs were used, based on ROC analysis, to determine sensitivity and specificity (see supplementary methods). The sensitivities to detect advanced adenomas was 21% (5/24, CI95% 9-40%) for PHACTR3 as well as for FIT. Combining PHACTR3 with FIT, meaning positive for at least one of the two measurements, increased the sensitivity to 33% (7/24, CI95% 18-53%). The sensitivities to detect CRC were 50% (10/20, CI95% 30-70%) for PHACTR3 and 65% (13/20, CI95% 43-82%) for FIT. Combining PHACTR3 with FIT increased the sensitivity to 95% (19/20, CI95% 76-99%). The specificity of the combination remained high (94% (45/48, CI95% 83-98%) compared to 96% (47/48, CI95% 86-99%) for PHACTR3 alone and 98% (47/48, CI95% 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 figure 4), 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 (CI95% 0.93-1.0) for CRC and 0.79 (CI95% 0.69-0.92) for advanced neoplasia (see figure 4), with sensitivity/specificity combinations of 61%/92%, 55%/96%, and 48%/98% (see table 2).
PHACTR3 methylation as stool DNA test for advanced adenoma and cancer separately). At these specificities, sensitivities for detecting CRC increased up to 15% using the combination of FIT and PHACTR3 methylation compared to FIT alone, although statistical significance was not reached.

Discussion

Stool-based DNA testing is an appealing approach for non-invasive 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 demonstrated promising performance in initial studies. In the present 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), thereby inhibiting the function of PP1(31), which in turn can trigger apoptosis and can inhibit oncogenic signaling due to its interaction with pRb(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 the present 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 out of 9 matched tumor tissues. While 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 to 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 demonstrated (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 re-expression 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 re-expression 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 - 66% for detecting CRC and a sensitivity of 32% for detecting advanced adenoma was observed at a specificity of 95-100%. The test performance of PHACTR3 can be further improved, which is illustrated by the higher AUC in tissues compared to stool, in particular the sensitivity to detect advanced adenomas. With the currently used method 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 two 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 the present 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, since 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. Since 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 performed 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, i.e. adenomas and CRC taken together, while maintaining a high specificity. These experiments were done in a series of stool sub-samples, 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 sub-samples were processed in a different manner compared to the whole stool samples described above, systematic differences exist when comparing these results to those from whole stool samples(4). Still, the results obtained demonstrate 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, the present 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.

Acknowledgements

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Reference List


(28) The Wellcome Trust Sanger Institute Cancer Genome Project web site 2011Available from: URL: http://www.sanger.ac.uk/genetics/CGP


Legends to the Figures

Figure 1. PHACTR3 methylation levels and mRNA expression and in cancer and matched normal tissues.

A. PHACTR3 methylation levels in tumor (T) and matched normal (N) tissues from nine CRC patients in (C). Quantifications represent mean methylation levels and (error bars correspond to standard deviation) relative to methylation levels in the normal tissues from three independent experiments. Methylation levels are calculated as (relative quantity of methylated PHACTR3 / relative quantity of unmethylated Beta-actin (ACTB)) ratio x 1000. Asterisks mark significant differences (p<0.05). B. mRNA expression analysis of PHACTR3 by RT-PCR in tumor (T) and matched normal (N) tissues from nine CRC patients. Quantifications represent mean expression values (error bars correspond to standard deviation) relative to expression levels in the normal tissue from three independent experiments.

Figure 2. PHACTR3 methylation levels in tissue and stool.

A. PHACTR3 methylation levels in tissue samples of 34 normal colon mucosa, 71 advanced adenomas and 64 carcinomas (UICC stage I to IV). B. PHACTR3 methylation levels in stool samples from 66 patients without colon neoplasia, 12 patients with hyperplastic polyps (HP) or non-advanced adenoma and 22 CRC patients (training set). Methylation levels are shown as (relative quantity of methylated PHACTR3 / relative quantity of unmethylated Beta-actin (ACTB)) ratio x 1000. C. PHACTR3 methylation levels in stool samples from 30 patients without colon neoplasia, 19 patients with advanced adenoma and 44 CRC patients (validation set). Methylation levels are shown as relative quantity of methylated PHACTR3 / relative quantity of unmethylated Beta-actin (ACTB)) ratio x 1000. Box plots show first quartile, median, third quartile and range of methylation levels. Dots represent individual data points, asterisks represent extremes.

Figure 3. Receiver operator characteristic analysis of PHACTR3 methylation in tissue and stool.

A. ROC curves of tissue samples. Sensitivity and specificity at various cut off values of PHACTR3 methylation in tissue samples from 64 carcinomas versus 34 normal colon mucosas (left panel) and
135 advanced neoplasia versus 34 normal colon mucosas (right panel). The areas under the curves are 0.93 and 0.95, respectively. **B.** ROC curves of stool samples (training set). Sensitivity and specificity at various cut off values of whole stool samples from 22 CRC patients versus 66 control patients without colon neoplasia (left panel) and 22 CRC patients versus 78 control patients (66 patients without colon neoplasia and 12 patients with hyperplastic polyps (HP) or non-advanced adenoma) (right panel). The areas under the curves are 0.78 and 0.77, respectively. **C.** ROC curves of stool samples (validation set). Sensitivity and specificity at various cut off values of whole stool samples from 44 CRC patients versus 30 control patients without colon neoplasia (left panel) and 63 patients with advanced neoplasia versus 30 control patients without colon neoplasia (right panel). The areas under the curves are 0.87 and 0.83, respectively. The solid line represents the ROC curve, the dashed line shows the reference line of no discrimination.

**Figure 4. Receiver operator characteristic analysis of FIT and PHACTR3 methylation in stool.**

**A.** Sensitivity and specificity at various cut off values of partial stool samples from 20 CRC patients versus 48 control patients without colon neoplasia for FIT, PHACTR3 methylation and for the combination of both tests. The areas under the curves are 0.92, 0.77, and 0.97, respectively. **B.** Sensitivity and specificity at various cut off values of partial stool samples from 44 patients with advanced neoplasia versus 48 control patients without colon neoplasia for FIT, PHACTR3 methylation and for the combination of both tests. ‘A’ represents the ROC curve for PHACTR3 methylation only, ‘B’ represents the ROC curve for FIT only, ‘C’ represents the ROC curve for the combination of FIT and PHACTR3 methylation. The areas under the curves are 0.76, 0.68, and 0.79, respectively.
Table 1. Test performances of PHACTR3, GATA4 and OSMR

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<th>PHACTR3</th>
<th>GATA4</th>
<th>OSMR</th>
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<tbody>
<tr>
<td><strong>Training set</strong></td>
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<tr>
<td>AUC [95% CI]</td>
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AA, Advanced adenoma; AUC, Area Under the Curve; CI, Confidence Interval; CRC, colorectal carcinoma; PHACTR3, Phosphatase and Actin Regulator; GATA4, GATA binding protein 4; OSMR, oncostatin M receptor
Table 2. Cut-offs and sensitivities of FIT, PHACTR3 methylation and their combination (Y) at equal specificities

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<th>Specificity</th>
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<td>sensitivity</td>
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</tr>
<tr>
<td>AA 98%</td>
<td>99.5</td>
<td>21%</td>
<td>1.5</td>
</tr>
<tr>
<td>CRC 98%</td>
<td>86.5</td>
<td>65%</td>
<td>1.1</td>
</tr>
<tr>
<td>Advanced Neoplasia</td>
<td>98%</td>
<td>41%</td>
<td>1.1</td>
</tr>
</tbody>
</table>

P-values are based on the comparison with FIT. AA, Advanced adenoma; CRC, colorectal carcinoma; FIT, Fecal Immunochemical Test; PHACTR3, Phosphatase and Actin Regulator 3;
Figure 2

A

PHACTR3 methylation level

normal mucosa n=34
advanced adenoma n=71
CRC Stage I n=21
CRC Stage II n=18
CRC Stage III n=20
CRC Stage IV n=5

B

PHACTR3 methylation level

controls n=66
HP/adenoma n=22
CRC n=22

C

PHACTR3 methylation level

controls n=30
advanced adenoma n=19
CRC n=44
Figure 3

A

- Tissue: CRC versus normal colon
  - AUC = 0.93
  - (95% CI = 0.87-0.98)

- Tissue: advanced neoplasia versus normal colon
  - AUC = 0.95
  - (95% CI = 0.93-0.98)

B

- Stool training set: CRC versus controls
  - AUC = 0.78
  - (95% CI = 0.64-0.91)

- Stool training set: CRC versus controls including non-advanced adenomas
  - AUC = 0.77
  - (95% CI = 0.64-0.90)

C

- Stool-validation set: CRC versus controls
  - AUC = 0.87
  - (95% CI = 0.79-0.95)

- Stool-validation set: advanced neoplasia versus controls
  - AUC = 0.83
  - (95% CI = 0.75-0.90)
Figure 4

CRC versus normal colon

- Curve A: Sensitivity = 0.77, Specificity = 0.92
- Curve B: Sensitivity = 0.92, Specificity = 0.97
- Curve C: Sensitivity = 1.0, Specificity = 1.0

Advanced neoplasia versus normal colon

- Curve A: Sensitivity = 0.68, Specificity = 0.76
- Curve B: Sensitivity = 0.76, Specificity = 0.79
- Curve C: Sensitivity = 1.0, Specificity = 1.0

AUC values:

- Curve A: 0.77
- Curve B: 0.92
- Curve C: 0.97
DNA methylation of phosphatase and actin regulator 3 detects colorectal cancer in stool and complements FIT

Linda J.W. Bosch, Frank A. Oort, Maarten Neerincx, et al.

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