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## Methylation of *TFPI2* in Stool DNA: A Potential Novel Biomarker for the Detection of Colorectal Cancer

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### Abstract

We have used a gene expression array–based strategy to identify the methylation of tissue factor pathway inhibitor 2 (*TFPI2*), a potential tumor suppressor gene, as a frequent event in human colorectal cancers (CRC). *TFPI2* belongs to the recently described group of embryonic cell Polycomb group (*PcG*)–marked genes that may be predisposed to aberrant DNA methylation in early stages of colorectal carcinogenesis. Aberrant methylation of *TFPI2* was detected in almost all CRC adenomas (97%,  $n = 56$ ) and stages I to IV CRCs (99%,  $n = 115$ ). We further explored the potential of *TFPI2* as a biomarker for the early detection of CRC using stool DNA–based assays in patients with nonmetastatic CRC and average-risk noncancer controls who were candidates for screening. *TFPI2* methylation was detected in stool DNA from stage I to III CRC patients with a sensitivity of 76% to 89% and a specificity of 79% to 93%. Detection of *TFPI2* methylation in stool DNA may act as a useful adjunct to the noninvasive strategies for screening of CRCs in the future.

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## Introduction

Epigenetic changes resulting in transcriptional silencing of cancer genes are among the most frequently discovered events in human tumor samples and are marked by aberrant accumulation of 5-methylcytosine and altered chromatin structure in the 5'-regulatory regions (1-3). Hypermethylation of gene promoter-associated CpG islands occurs early during colorectal cancer (CRC) development (4), suggesting its utility as a molecular marker for both early detection and monitoring progression of the disease.

CRCs, although amenable to screening and early detection, still remains the second leading cause of cancer-related mortality in the United States (5). Current screening modalities have resulted in only a modest decrease in mortality and failed to achieve high public participation (<50%; refs. 6,7). Strategies such as colonoscopy are invasive whereas stool occult blood test has limitations of repeat measurements and interference by dietary components. Stool DNA tests are noninvasive, may have high specificity with single measurements, and may achieve more extensive sampling of colon and has recently been recommended for CRC screening (8,9). The stool DNA tests tested thus far are frequently based on multiple genetic markers including *KRAS*, *APC*, *TP53*, and *BAT26*, which may be more costly and difficult to implement (10). Epigenetic events which are seen more frequently than genetic events (11) may have a potential to achieve high sensitivity and specificity using a single gene (9).

We have recently described a novel expression array-based approach to identify the CRC "DNA hypermethylome" estimated to contain ~400 to 600 hypermethylated genes per individual tumor (11). Many of these genes may already be marked for transcriptional repression in embryonic precursor cells by the PcG proteins (11-13), which participate in the three-dimensional structure of nuclear DNA (14) and may target genes with a characteristic "bivalent" promoter chromatin structure containing both active and repressive histone modifications. Such PcG-marked genes are poised to integrate transcriptional signaling during cellular proliferation and differentiation (15). Moreover, such PcG-marked genes may be predisposed to methylation early on in carcinogenesis (12,13), and such genes may be good targets for investigation as biomarkers for early detection.

One gene that we have identified within the DNA hypermethylome, residing at the intersection of both the hypermethylome and PcG-marked genes, is tissue factor pathway inhibitor (*TFPI2*), a Kunitz-type serine proteinase inhibitor that protects the extracellular matrix of cancer cells from degradation and inhibits *in vitro* colony formation and proliferation (16,17). It is thought that loss of *TFPI2* function could predispose cells toward a proinvasive program, consistent with an important role for this protein in later stages of carcinogenesis.

In this study, we investigate the role of *TFPI2* in CRC cell lines and tumors. We present data indicating that *TFPI2* methylation is a frequent and early event in CRCs. Furthermore, we show that *TFPI2* methylation in stool DNA is a potential biomarker for noninvasive detection of colorectal neoplasms.

## Materials and Methods

### Gene Expression, Methylation Analysis, and Chromatin Immunoprecipitation

Cell culture growth, maintenance, pharmacologic treatment and expression array analysis were performed as previously described (11). Total RNA was isolated as previously described (11) or purchased from Stratagene (normal adult colon). cDNA synthesis and reverse transcriptase PCR (RT-PCR) were performed as described (11). Quantitative RT-

PCR was performed with Quantitect SybrGreen PCR Mix (Qiagen) using an iCycler (Bio-Rad). DNA isolation, bisulfite modification, primer design, and methylation analysis were performed as previously described (11). Complete primer sequences and thermocycler variables are shown in Supplementary Table S1. ChIP on Chip experiments and analyses were performed as previously described (18).

### Colony Formation Assay and *In vitro* Growth Rate

We amplified the complete 820 nucleotide *TFPI2* coding region with primers 5-ACGA-GCTAGC-GCACCATGGACCCCGCTCG-3 (*Nhe* I in bold-face) and 5-AGCT-GAATTC-TGTTTAAAATTGCTTCTTCCG-3 (*Eco* RI in boldface) from normal human colon cDNA (Stratagene), purified, restriction enzyme digested, and cloned into the *Nhe* I and *Eco* RI site of pIRES-Neo3 (Invitrogen). Transfection, colony formation, and scoring were as previously described (11). *In vitro* growth rate was performed using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. MTT assay was performed in HCT116 cell lines transfected with plasmid carrying the *TFPI-2* gene (1 µg/µL), or empty vector (1 µg/µL) alone as a control. The ATCC-MTT cell proliferation assay kit was used and all experiments were performed as previously described (19).

### Primary Human Tissue Samples

Primary tissue samples were obtained from the archives of the Department of Pathology, Johns Hopkins University with Institutional Review Board approval and Health Insurance Portability and Accountability Act compliance. This study compiles data from formalin-fixed and paraffin-embedded primary CRCs of tumor stages I to IV ( $N = 115$ ;  $n = 35$  stage I,  $n = 36$  stage II,  $n = 28$  stage III,  $n = 16$  stage IV; mean age, 65.2 years) and corresponding adjacent nonneoplastic colon tissue in a subset of 22 of these CRC patients ( $n = 22$ ), colorectal adenomas from patients without an associated invasive CRC ( $N = 56$ ;  $n = 17$  serrated,  $n = 17$  tubular,  $n = 22$  villous) and normal colon controls of patients without any colorectal neoplasms ( $n = 48$ ). Within this subset of normal colon controls, 16 samples were age-matched for CRCs (mean age, 63.4 years;  $P =$  not significant). For expression analysis, fresh-frozen CRCs ( $n = 12$ ) and corresponding adjacent normal colon tissue ( $n = 3$ ) were used.

### Stool Studies

**Study population and sample size**—Colonoscopy-negative control stool samples were obtained from healthy, average-risk subjects >50 years of age who were screened for CRC within the framework of a workplace-based community CRC study at the Maastricht University Medical Center. Stool samples from colonoscopy-confirmed CRC/adenoma patients covering all CRC stages and matched CRC tissue were collected at the VU University Medical Center in Amsterdam. The Medical Ethical Committee of the Maastricht University Medical Center and the Dutch Health Council approved this study. Written informed consent was obtained for all stool samples. All control stool samples and a subset of the CRC stool samples were collected within 2 weeks prior to colon purgation and colonoscopy. Some CRC stool samples were collected 5 to 7 days following colonoscopy. All stool samples were homogenized in stool stabilization buffer (Exact Sciences), aliquoted in portions containing the equivalent of 4 g and processed within 48 h after defecation. Stool samples were handled and analyzed in a blinded fashion during storage, DNA isolation, and PCR analysis. A pilot feasibility study with a sample size of 30, including 18 cases (11 CRCs and 7 adenomas) and 12 controls, was performed to investigate the potential utility of *TFPI2* methylation in stool DNA for the detection of CRC. Following the pilot feasibility study, a double blind study with a training set and validation set was designed. The training set consisted of 71 samples whereas the validation set consisted of 96 samples. The training

set included 26 stage I to III CRCs and 45 controls. The validation set, which had a sample size of 96, included 66 cases and 30 controls. Cases in the validation set targeted the screen-relevant neoplasms including advanced adenomas (i.e., >1 cm size, high-grade dysplasia, or villous histology;  $n = 19$ ) and stage I to III CRCs ( $n = 47$ ). Figure 1 depicts the schematic representation of the study design.

### Stool Sample Collection and Processing

**Stool DNA extraction**—Samples were homogenized in excess volume (1:7) of stool homogenization buffer (Amresco) and aliquoted in portions of 32 mL (containing ~4 g of stool). Single aliquots were centrifuged and the supernatants were incubated with RNase A for 60 min at 37°C. For the pilot feasibility stool DNA studies, total DNA was precipitated with sodium acetate (pH 5.2)-isopropanol, washed with 70% ethanol and resuspended in 4 mL of 1× TE (pH 7.4). Following the addition of 400 μL of 10× buffer [240 mmol/L EDTA (pH 8.0), 750 mmol/L NaCl], 400 μL of 10% SDS, and 20 μL of proteinase K (20 mg/mL), samples were incubated for 16 h at 48°C with shaking. After the addition of 5 mL of phenol/chloroform/isoamyl alcohol, the samples were incubated for 10 min at room temperature before centrifugation. The aqueous layer was transferred to a new tube, DNA was precipitated, washed, and pellets were resuspended in 2 mL of LoTE (pH 8.0).

The stool DNA extractions for the training and validation studies were performed using the QIAamp DNA stool mini kit protocol for ease of DNA extraction. The initial homogenization steps are similar. After RNase A treatment, total DNA was precipitated and resuspended in 1× TE. Half of this resuspended DNA was then used as input for the QIAamp DNA stool midi test kit (Qiagen, user-developed protocol). ASL buffer (1.5 mL) and an InhibitEX tablet were added and the mixture was centrifuged. Next, 150 μL of proteinase K was added to 2 mL of supernatant, which was mixed with 2.4 mL of buffer AL and incubated for 10 min at 70°C. Ethanol (2 mL) was added and loaded onto the column in portions of 3.3 mL maximum. After a double washing step, the column was dried and DNA was eluted using 200 μL of buffer AE.

### Stool DNA Methylation Studies

**Pilot feasibility study**—Genomic DNA (2 μg) was modified using Zymo Kit according to the instructions of the manufacturer. A nested strategy, as previously described, was used as DNA obtained from stool is limited in amount (20). An initial unbiased amplification of bisulfite-modified DNA was done using external primers located in the flanking region for 35 cycles. Each PCR was carried out in a volume of 25 μL with 1 μL of JumpStart Red Taq DNA polymerase (Sigma), 10 pmol of each external primer, and 4 μL of bisulfite-modified DNA. PCR conditions included an initial denaturation at 95°C for 5 min followed by 35 cycles of (95°C × 30 s, annealing temp × 30 s, 72°C × 30 s) and final elongation at 72°C for 5 min. In the second stage, 1:1,000 dilutions of first-stage products were used and reamplified for 30 cycles using an internal primer set. Except for the annealing temperatures and cycle number, PCR cycling conditions were the same for the first and second stages. Primer sequences are provided in Supplementary Table S1.

For the training and validation sets, a quantitative approach was pursued. Quantitative methylation-specific PCR was performed using a 7900HT real-time PCR system (Applied Biosystems). DNA (2.4 μL) was added to a PCR mix containing buffer [16.6 mmol/L (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 67 mmol/L Tris, 6.7 mmol/L MgCl<sub>2</sub>, 10 mmol/L β-mercaptoethanol], deoxynucleotide triphosphates (5 mmol/L), forward primer (6 ng/μL), reverse primer (18 ng/μL), molecular beacon (0.16 μmol/L), BSA (0.1 μg), and Jumpstart Taq polymerase (0.4 units; Sigma Aldrich). The PCR program was as follows: 5 min at 95°C, followed by 45 cycles of 30 s at 95°C, 30 s at 57°C, and 30 s at 72°C, followed by 5 min at 72°C. A

standard curve ( $2 \times 10^6$ – $20$  copies) was included to determine copy numbers of unknown samples by interpolation of their Ct values to the standard curve. Primer and beacon sequences are provided in Supplementary Table S1.

### Statistical Analysis

Statistical analysis was performed using the STATA 9.2 software package. Receiver operator characteristic (ROC) curve analysis, using the area under the curve, was used to determine the best cutoff value for the highest sensitivity and specificity in the training set. Wherever mean ages of the cases and controls differed significantly, a ROC-GLM regression model was used to assess the accuracy of *TFPI2* promoter methylation after adjustment for age (21). *TFPI2* methylation was considered positive if a methylation value was higher than the cutoff (10 copies).

### Results

The *TFPI2* gene showed a ~50-fold increase in gene expression in HCT116 (Fig. 2A) after 5-aza-2'-deoxycytidine (DAC) treatment in our array-based hypermethylome analyses (11), generating further interest in the potential role of this gene in CRCs. We first tested *TFPI2* expression by RT-PCR (Fig. 2B, top) and DNA methylation by MSP (Fig. 2B, bottom) before and after DAC treatment in HCT116 colon cancer cells. Consistent with our microarray results, *TFPI2* was silenced and methylated in untreated HCT116 cells and exhibited re-expression with concurrent demethylation of the *TFPI2* promoter (Fig. 2B and C) after drug treatment with DAC. Bisulfite sequencing of a region spanning the promoter from -286 bp to +76 bp confirmed dense methylation of *TFPI2* in HCT116, whereas DKO a control cell line, harboring almost no cytosine methylation, showed a complete absence of methylation (Fig. 2C, bottom), again consistent with the microarray data (Supplementary Fig. S1). These results suggest an association between *TFPI2* promoter methylation and expression in HCT116 cells, as predicted by our hypermethylome array analyses. Additionally, *TFPI2* promoter showed an enrichment peak for the repressive histone mark, H3K27me3, along with an enrichment peak for the active mark, H3K4me2, over the transcription start site in HCT116 cells, where it is methylated and silenced. Although in DKO cells, where *TFPI2* is unmethylated and expressed, there is a further increase in enrichment of H3K4me2, accompanied by rearrangement of this mark to now exhibit a dip at the transcription start site, a chromatin pattern that characterizes DNA-hypermethylated genes in colon cancer cells (Fig. 2D; ref. 18). These data show the bivalent chromatin structure marked by the presence of both active and repressive histone marks near the *TFPI2* promoter in cancer cells consistent with its identification as a PcG target gene (22-24).

We further tested the expression of *TFPI2* in a panel of CRC cell lines. Scatter plot analyses indicated significant re-expression of *TFPI2* after DAC treatment in SW480 (6.9-fold) and RKO (19.3-fold) cells, consistent with data obtained in HCT116 cells. However, only a modest re-expression (1.5-fold) was seen in HT29 cells (Fig. 3A). RT-PCR (Fig. 3B and C) and MSP (Fig. 3D) confirmed re-expression of *TFPI2* and concomitant demethylation, following DAC treatment, in nearly all CRC cell lines (Fig. 3D). Methylation of *TFPI2* was also detected in other tumor cell lines including gastric, esophageal, pancreatic, and breast cancer cell lines (Supplementary Fig. S2).

We next tested for the methylation of *TFPI2* in normal colonic tissue from cancer-free patients sorted by age (Fig. 4A, top; and Fig. 4B). *TFPI2* methylation was seen infrequently in normal colon from noncancerous patients (3 of 48; 6.2%; Fig. 4). Within this subset of normal colon controls, 16 samples were age-matched for the CRCs (mean age, 63.4 years;  $P$  = not significant), and methylation was observed only in this subset of patients. However, surrounding nonneoplastic colonic tissue of patients with CRC showed much more frequent

*TFPI2* methylation (12 of 22; 54%; Fig. 4A, top; Fig. 4B). Interestingly, *TFPI2* methylation was observed in 94% of serrated adenomas (16 of 17), 100% of tubular adenomas (17 of 17), and 100% of villous adenomas (22 of 22), all of which represent preinvasive stages of colorectal carcinogenesis and in 99% of invasive CRC stages I to IV (114 out of 115; Fig. 4A, middle and bottom; Fig. 4B). Bisulfite sequencing of *TFPI2* showed dense DNA methylation in primary tumor samples (Fig. 4C). Interestingly, the expression of *TFPI2* in fresh-frozen primary samples also correlates with promoter DNA methylation status. We found a lack of *TFPI2* expression in virtually all CRC samples (11 out of 12 CRC samples), and RT-PCR analysis of three paired normal cancer samples show that the gene is still expressed in surrounding normal colon tissue (Fig. 4D, top; the comparative DNA methylation status of these samples is shown in Fig. 4D, bottom).

We also performed studies to detect the putative tumor suppressor role of *TFPI2* in CRC tumorigenesis. We used *in vitro* colony formation assays to determine the effects of full-length *TFPI2* transfected into HCT116 cells lacking *TFPI2* expression. Overexpression of full-length *TFPI2* induces a nearly 10-fold reduction in colony number of HCT116 cells, with surviving clones showing a severely depleted size, comparable to results obtained with the bona fide tumor suppressor *p53* (Fig. 5A and B). These studies suggest a putative tumor suppressor role for *TFPI2* in CRC. MTT assay showed a modest decline in cell growth in *TFPI2*-transfected cells as compared with empty vector ( $P =$  not significant). There was no significant difference in cell growth in the mock-treated and empty vector-treated cells. Supplementary Fig. S3 shows the results for MTT assay in detail.

All of the above data indicate that *TFPI2* is a potential tumor suppressor gene which is expressed and unmethylated in colonic epithelium from cancer-free individuals, and early methylation may occur during carcinogenesis. With this in mind, we tested the potential viability of using *TFPI2* DNA methylation to detect CRC in stool DNA. We conducted a pilot feasibility study with a blinded case-control design to analyze stool DNA extracted from 30 patient samples: 18 cases  $\rightarrow$  mean age = 58.5 years, including 11 patients with CRC and 7 patients with adenomas with dysplasia; and 12 controls  $\rightarrow$  mean age = 54.2 years (Table 1A). 60% of the subjects were males whereas 40% were females. In 8 of 11 stool samples from patients with CRC and 3 of 7 samples from patients with preinvasive neoplastic lesions we detected methylated *TFPI2* alleles whereas none of 12 (0%) samples of control patients were positive for methylated *TFPI2* (sensitivity, 73%; specificity, 100% for cancers; sensitivity, 43%; specificity, 100%, for adenomas; Table 1B).

After the promising initial pilot feasibility study, we further verified the utility of *TFPI2* stool DNA methylation for detection of CRC using a training and validation set. Initial pilot feasibility study had a limitation of using phenol chloroform extraction protocol for DNA extraction from stool which is a time-consuming and labor-intensive process. Envisioning the potential utility of *TFPI2* to design stool assays for screening purposes in the future, we used QIAamp DNA extraction stool mini kit protocol which is a faster and more efficient platform for DNA extraction and similarly adapted to a real-time platform. Methodologies and analyses were consistent for the training set and validation set. In the training set, overall mean age for all subjects including cases and controls was 60.5 years. Mean ages for stage I to III CRCs and controls were 69.3 and 55 years, respectively (Table 1A); 52% of the subjects were males whereas 46% were females (Table 1A). In the training set, *TFPI2* stool DNA methylation had a sensitivity of 89% for detection of stage I to III CRCs with a specificity of 79% (Table 1B). Because the mean age of the cases and controls differed, a ROC-GLM regression model was used to assess the accuracy of *TFPI2* promoter methylation after adjustment for age. This analysis indicated that age did not significantly influence the accuracy ( $P = 0.58$ , ROC-GLM regression model). In the validation set, mean age for all subjects including cases and controls was 63.3 years. Mean ages for cases and

controls were 68.3 years (71.1 years for CRC and 61.4 years for adenomas) and 52.3 years, respectively. 45% of the study subjects were males whereas 54% were females (Table 1A). In the validation set, *TFPI2* stool DNA methylation had a sensitivity of 76% for detection of stage I to III CRCs with a specificity of 93%. However, only 4 out of 19 advanced adenomas (sensitivity 21%) could be detected with a specificity of 93%. Table 1B summarizes the results in detail.

## Discussion

Previous studies have shown *TFPI2* hypermethylation in other cancer types (16,17,25-28), suggesting an important role for this gene in the etiology of human malignancies. Based on the high level of gene re-expression measured in our array analysis, frequent methylation and silencing of *TFPI2*, which responded to DAC treatment in CRC cell lines, and the presence of the PcG mark, which predisposes genes to methylation early on during carcinogenesis, we speculated that *TFPI2* might represent a candidate for the detection of CRC. We detected the methylation of *TFPI2* in nearly all primary adenomas and CRC samples. For stool DNA tests, the sensitivity was found to be 76% to 89% for stage I to III CRCs, with a specificity of 79% to 93%. Evidence from our study and others indicate that *TFPI2* may be an important tumor suppressor gene in CRC and other malignancies (16,17,25-28).

Our present findings have potential clinical implications. To date, it seems that the most reliable early detection of cancer is provided by a signature combining multiple gene mutations, each of which individually has a low sensitivity (10,29,30). This is further supported by the current genome-wide sequencing efforts which have shown that each of these mutational event is seen in a small number of tumors (<15-50%; ref. 31). Interestingly, some of these studies have reported high sensitivity for stool DNA-based mutational assays but did not comment on specificity (32). Nonetheless, these studies have played an important role in spreading the idea of stool DNA-based tests and provided insightful data on mutations present in the stool DNA of CRC patients. Detection of CRC-specific methylation in stool samples using a single epigenetic marker has also been explored recently (33-35). Ultimately, the goal is to design an ideal screening tool which is noninvasive, sensitive, specific, cost-effective, and easy to implement across large populations. This is particularly of importance, given the recent guidelines by the American Cancer Society, recommending stool DNA testing for CRC detection (8).

Epigenetic studies which focus on analyzing single genes may be more cost-effective for screening. In general, the available results of single marker epigenetic studies are difficult to compare because study designs and patient populations differ. However, these studies have provided impressive preliminary data about these single epigenetic markers and their potential utility as stool DNA-based assays. Zhang and colleagues reported a sensitivity of 86% and specificity of 89% for *SFRP1* using a cohort of 36 patients, of which 9 had undergone neoadjuvant therapy and most cases (31 of 36, 88%) were predominantly rectal or left-sided (35). Our results are not comparable with theirs as they had a smaller sample size, no validation cohort, lacked a blinded study design, and included stage IV patients and patients who had undergone neoadjuvant chemotherapy. Moreover, inclusion of rectal adenomas and predominantly rectal and left-sided carcinomas may result in higher sensitivity. Other single marker methylation-based stool studies using *Vimentin* (33) and *SFRP2* (34,36,37) have reported a sensitivity of 52% and 87% to 94%, respectively, and a specificity of 90% and 85% to 90%, respectively. Muller and colleagues reported a sensitivity of 77% to 90% and a specificity of 77% for *SFRP2* in a set of 49 patients (38). Some of these studies included a substantial number of stage IV patients or did not mention the stage of tumors (34,36). Conversely, the strengths of our study include its double-blinded design, exclusion of stage IV patients, and independent training and validation sets

which were lacking in previous studies. Another interesting finding is the discovery of frequent methylation of *TFPI2* in >95% of colorectal adenomas and cancers which has not been investigated earlier. Our data indicate that *TFPI2* stool DNA methylation has a sensitivity of 76% to 89% for the detection of stage I to III CRCs and specificity of 79% to 93%. However, for adenomas, although >95% methylation was seen in primary tissues, only 21% to 43% sensitivity was achieved for stool samples. This could potentially be explained by fewer cells shed by adenomas and the lack of high potential for anchorage-independent growth in the shed adenoma cells, which could have resulted in their degradation (9). Other stool DNA methylation-based studies have reported a sensitivity of 42% to 77% for the detection of CRCs and a relatively low sensitivity of 31% to 48% for the detection of adenomas with a specificity of 73% to 100% for both CRCs and adenomas (33-35,39).

Although a total of 197 stool DNA samples were analyzed in the current study, the sample size is still smaller as compared with multicenter trials and there is a need for studies with larger sample size for cost-effective analyses and better measurement of sensitivity and specificity. Another limitation is the lack of methylation data on tumors of patients whose stool was analyzed in the current study. This may have provided better correlation and understanding of factors which may have resulted in reduced sensitivity for adenomas.

In conclusion, *TFPI2* methylation is an early and frequent event in colorectal adenomas and carcinomas which is seen infrequently in the mucosa of normal noncancer patients. We have reported, for the first time, the bivalency of the *TFPI2* promoter and its potential role as a tumor suppressor in CRC. Our data on stool DNA of CRC and adenoma patients suggests that *TFPI2* promoter methylation is a feasible epigenetic marker for the detection of CRC and may be useful for CRC screening in future.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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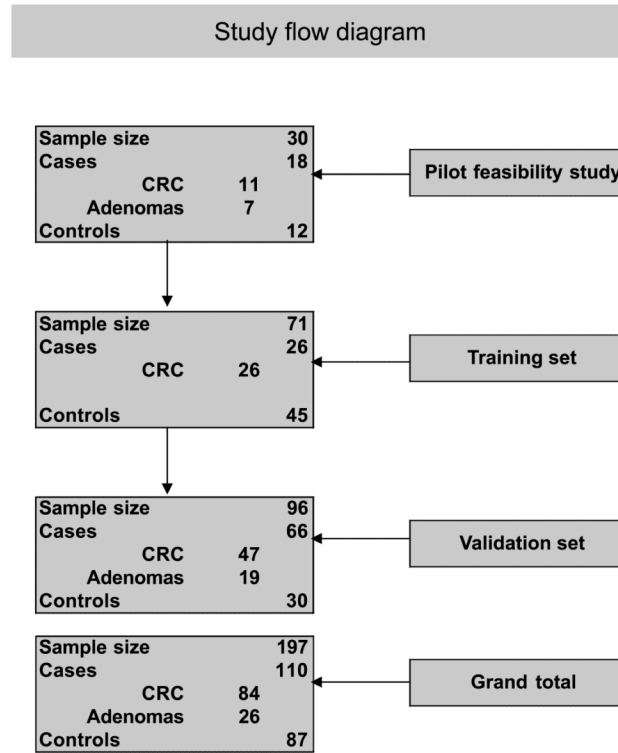
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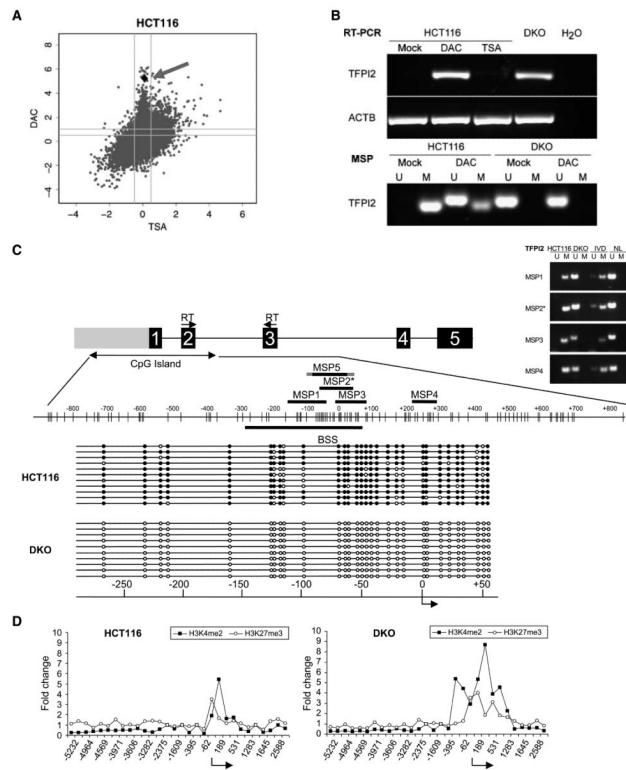
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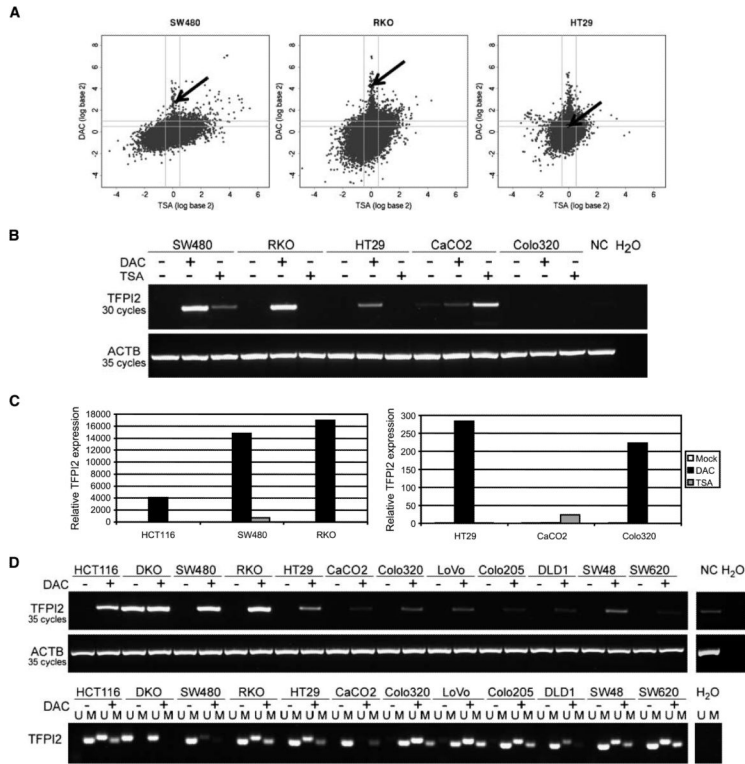


**Figure 1.**

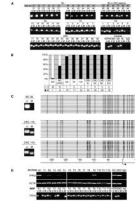
Schematic representation of the stool DNA study design: 30 samples including 11 CRCs, 7 adenomas, and 12 controls were analyzed in the initial pilot study. Independent training and validation set with sample sizes of 71 and 96, respectively, were then further tested.

**Figure 2.**

Epigenetic inactivation of *TFPI2* in CRC cell line HCT116. **A**, gene expression changes are plotted by fold change (log scale) in HCT116 after DAC (*Y*-axis) or trichostatin A (*TSA*; *X*-axis) treatment. *TFPI2* expression increases 5.24 in log<sub>2</sub> ratio (37.7-fold) in HCT116 after DAC treatment compared with HCT116 Mock and shows no response to *TSA*. ◆ and ↑, location of *TFPI2* in the scatter plot. **B**, expression status (*top*) of *TFPI2* in HCT116 without any treatment (*Mock*) and after DAC or *TSA* treatment, respectively, versus DKO. MSP analyses (*bottom*) in cell lines without (*Mock*) and after DAC treatment for unmethylated (*U*) and methylated (*M*) DNA. **C**, MSP tiling of the *TFPI2* promoter and exon 1 along -118 bp to +284 bp from TSS using four different MSP primer sets (*MSP1–4*) shows unequivocal methylation status (*top*). *IVD*, *in vitro* methylated DNA, positive control; *NL*, normal lymphocytes from healthy individuals; *TFPI2* gene map with the promoter region (*gray box*), and subsequent genomic sequence including exons 1 to 5 (*black boxes*). The primer binding sites for MSP and BSS are indicated along the CpG island (*middle*). The *MSP2* primer set was used for methylation studies in primary formalin-fixed and paraffin-embedded and fresh-frozen tissues. *MSP5* primer set was used for nested MSP analysis for fecal DNA methylation studies. Bisulfite sequencing of the *TFPI2* promoter in HCT116 and DKO cells (*bottom*). ○, unmethylated CpG residues; ●, methylated CpG residues; arrow, transcription start site. **D**, histone marks for H3K4me2 and H3K27me3 at the *TFPI2* promoter of HCT116 (*left*) and DKO (*right*) cells as determined by ChIP on ChIP assay.

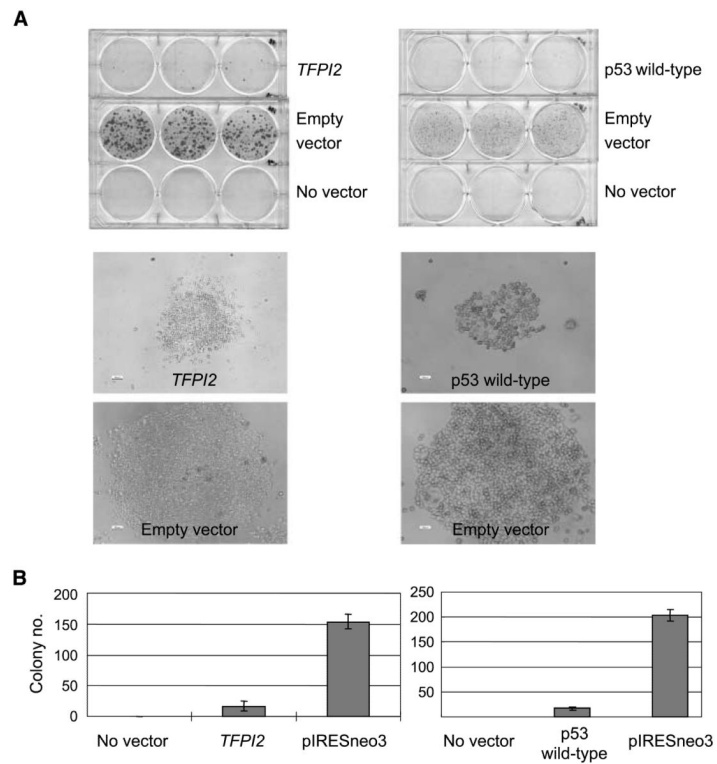


**Figure 3.** Epigenetic inactivation of *TFPI2* in CRC cell lines. *A*, gene expression changes for the indicated cell lines are plotted by fold change (log scale) after DAC (*Y*-axis) or TSA (*X*-axis) treatment. ◆ and ↑, the location of *TFPI2*. *B*, RT-PCR results for *TFPI2* in CRC cell lines without any (-) and after DAC or TSA (+) treatment, respectively, as indicated. Normal colon (*NC*) was used as positive control for *TFPI2* expression. β-Actin (*ACTB*) was used as control for equal amplification. *C*, real-time RT-PCR results for HCT116, SW480, and RKO (*left*) and HT29, CaCO2, and Colo320 (*right*) without any (*Mock*) and after DAC or TSA treatment, respectively. Please note the differing scale used for the two graphs. *White columns*, mock; *black columns*, DAC; *gray columns*, TSA. *D*, expression (*top*) and MSP status (*bottom*) for various CRC cell lines before (-) and after (+) DAC treatment (1 μmol/L, 72 h).



**Figure 4.**

Epigenetic inactivation of *TFPI2* in primary human tissues. *A*, methylation status by MSP in representative samples of normal colon (*NC*), adjacent normal colon from CRC patients, adenomas (serrated, *SA*; tubular, *TA*; villous, *VA*), and CRCs. For normal colons, note the age in years (*y*) as indicated in shaded box. *U*, unmethylated; *M*, methylated; *NL*, normal lymphocytes. *B*, bar graph showing MSP results of all primary samples ( $n = 230$ ). *C*, bisulfite sequencing results for *TFPI2* of one normal colon (*NC 35*) and two representative CRC samples (*CRC 116* and *CRC 119*), with the location relative to the TSS as indicated. ● methylated CpG, ○, unmethylated CpG. *Insets*, methylation status by MSP. *D*, *TFPI2* expression and methylation status in fresh-frozen primary human CRCs (*T1–12*) and corresponding normal colon (*N1–3*).



**Figure 5.**

A, colony formation assay in HCT116 cells transfected with either full-length *TFPI2* (left) or p53 (right) versus corresponding empty vector (middle) or no vector control (bottom) after hygromycin selection (top). Resulting colonies show a significant decrease in colony size in HCT116 with *TFPI2* (left) as well as p53 (right) containing vector versus corresponding empty vector below (bottom). B, bar graph representing quantification of colony number after transfection of the indicated plasmids.

**Table 1**

## Patient characteristics

<b>(A) Study population characteristics</b>			
	<b>Pilot study</b>	<b>Training set</b>	<b>Validation set</b>
Number of study subjects ( <i>N</i> )	30	71	96
Age ( <i>y</i> )		Mean ( <i>y</i> )	
CRC	60.7	69.3	71.1
Adenoma	55.1		61.4
Controls	54.2	55	52.3
Sex		Percentage	
Male	60	52	45
Female	40	46	54
Unknown		2	1
<b>(B) Summary of test performance</b>			
	<b>Pilot study (<i>n</i> = 30)</b>	<b>Training set (<i>n</i> = 71)</b>	<b>Validation set (<i>n</i> = 96)</b>
CRC	<i>n</i> = 11	<i>n</i> = 26	<i>n</i> = 47
Sensitivity (95% CI)	73 (39–94)	89 (70–98)	76 (60–88)
Specificity (95% CI)	100 (74–100)	79 (64–90)	93 (77–99)
PPV (95% CI)	100 (63–100)	72 (53–86)	94 (80–99)
NPV (95% CI)	80 (52–96)	92 (78–98)	73 (56–86)
Adenoma	<i>n</i> = 7		<i>n</i> = 19
Sensitivity (95% CI)	43 (10–82)		21 (6–46)
Specificity (95% CI)	100 (74–100)		93 (78–99)
PPV (95% CI)	100 (29–100)		67 (22–96)
NPV (95% CI)	75 (48–93)		65 (49–79)

Abbreviations: PPV, positive predictive value; NPV, negative predictive value; 95% CI, 95% confidence interval.