High-throughput DNA methylation analysis in colorectal cancer and childhood leukemia

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Chapter 2

Tumour-specific methylation of *PTPRG* intron 1 locus in sporadic and Lynch syndrome colorectal cancer

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TUMOUR-SPECIFIC METHYLATION OF *PTPRG* INTRON 1 LOCUS IN SPORADIC AND LYNCH SYNDROME COLORECTAL CANCER

Abstract

DNA methylation is a hallmark in a subset of right-sided colorectal cancers. Methylation-based screening may improve prevention and survival rate for this type of cancer, which is often clinically asymptomatic in the early stages. We aimed to discover prognostic or diagnostic biomarkers for colon cancer by comparing DNA methylation profiles of right-sided colon tumours and paired normal colon mucosa using an 8.5k CpG island microarray. We identified a diagnostic CpG-rich region, located in the first intron of the *PTPRG* gene, with altered methylation already in the adenoma stage, i.e. prior to the carcinoma transition. Validation of this region in an additional cohort of 103 sporadic colorectal tumours and 58 paired normal mucosa tissue samples showed 94% sensitivity and 96% specificity. Interestingly, comparable results were obtained when screening a cohort of Lynch syndrome-associated cancers. Functional studies showed that *PTPRG* intron 1 methylation did not directly affect *PTPRG* expression, however, the methylated region overlapped with a binding site of the insulator protein CTCF. Chromatin immunoprecipitation (ChIP) showed that methylation of the locus was associated with absence of CTCF binding. Methylation-associated changes in CTCF binding to *PTPRG* intron 1 could have implications on tumour gene expression by enhancer blocking, chromosome loop formation or abrogation of its insulator function. The high sensitivity and specificity for the *PTPRG* intron 1 methylation in both sporadic and hereditary colon cancers support biomarker potential for early detection of colon cancer.

Introduction

DNA methylation is a common mechanism in colorectal tumorigenesis[1, 2]. Over the last decade, several genome-wide array-based methods have been developed, allowing the discovery of novel tumour-specific methylated loci. Enzymatic (HELP[3], MMASS[4], DMH[5], CHARM[6]) and ChIP methods[7] are most commonly used for genome-wide screening of DNA methylation, in combination with CpG island or promoter microarrays. An alternative genome-wide approach to identify genes silenced by DNA methylation detects expression differences in cell lines treated with DNA demethylating agents[8, 9]. More recently, captured methylated DNA[10] and bisulphite-converted reduced representations[11] are analyzed by high-throughput sequencing strategies. The unbiased approaches have indicated that transcription regulation associated with CpG methylation is not restricted to promoter CpG islands[12, 13]. Conserved regions up to 2kb distant from the promoter, annotated as CpG island shores[12], and promoter CpG islands of lesser density, annotated as intermediate-CpG islands[13], undergo cancer-specific methylation more often than traditional promoter CpG islands. The methylation status of these regions is strongly related to gene expression and might have been underestimated in previous studies.

The aim of this study was to discover novel tumour-specific DNA methylation markers

in right-sided colon cancer. These tumours have a higher frequency of the CpG island methylator phenotype (CIMP). Additionally, right-sided tumours are often clinically asymptomatic at early stages, thus, patients would greatly benefit from a reliable screening method. We employed differential methylation hybridization (DMH) combined with a 8.5k CpG clone library microarray for the initial identification of differential methylation in a cohort of colon cancers[14]. This library is enriched for CG-rich areas throughout the genome, encompassing promoter CpG islands as well as CpG-rich island shores and intermediate-CpG islands[15]. We report tumour-specific methylation of the first intron of the receptor protein-tyrosine phosphatase gamma gene (*PTPRG*), in both sporadic and Lynch syndrome colorectal cancers. Additionally we demonstrate that methylation of this region affects its binding to the CCCTC-binding factor (zinc finger protein, CTCF).

Materials and Methods

Tissue

Anonymized samples were obtained from patients who underwent surgery between 1988 and 2006 at the Leiden University Medical Center (Leiden, The Netherlands) or at the Rijnland Hospital (Leiderdorp, The Netherlands). Tumour sections were micro-dissected to minimize normal epithelium and stromal cells. DNA was isolated from fresh-frozen tissue using a previously described method[16], and from formalin-fixed paraffin-embedded (FFPE) tissue using the Wizard Genomic DNA Purification kit (Promega, Madison, WI, USA). We used available normal mucosa from the same individuals as control to correct for age-dependent methylation. Age, location and microsatellite instability (MSI) status for the sporadic tumours are listed in Supplementary Table S1, and for the Lynch syndrome-associated tumours in Supplementary Table S2. The colorectal cancer cell lines SW48, RKO, SW480, Caco2, SW837, and LS411 were obtained from the American Type Culture Collection (Manassas, VA, USA). DNA was isolated from these cell lines as described previously[16]. RNA was isolated using TRIZOL (Invitrogen, Carlsbad, CA, USA) and subsequently purified with Qiagen RNeasy columns combined with the RNase-free DNase kit (Qiagen Sciences, Germantown, MD, USA). The present study was approved by the Medical Ethics committee of the LUMC (protocol P01-019). Cases were analyzed following the medical ethical guidelines described in the Code Proper Secondary Use of Human Tissue established by the Dutch Federation of Medical Sciences.

CpG island microarrays

CpG island clone inserts (8544) were amplified using vector-based primers as described previously[14, 17]. The CpG island clone library was originally generated by the Sanger Centre from affinity-purified *in vitro* methylated DNA fragments[15]. Clone sequence information was downloaded from the Toronto Microarray Facility. PCR products were spotted onto CodeLink (GE Healthcare, Munich, Germany) slides using an OmniGrid arrayer (Genomic Solutions, Ann Arbor, MI, USA) at the Leiden Genome Technology Center (www.lgtc.nl) as described[18].

Differential methylation hybridization

DMH was performed according to Yan *et al.*[14]. Cy5-labeled amplicons, representing methylated DNA fragments derived from tumours and paired normal mucosa samples,

were co-hybridized to the CpG island microarrays with a Cy3-labeled common reference amplicon consisting of a pool of DNA from the six colorectal cancer cell lines described above. Detection was done on a G2565BA scanner (Agilent Technologies, Santa Clara, CA, USA) and image analysis using GenePix6.0 (Molecular Devices, Union City, CA, USA). Pre-processing, including normalization, was performed using the GenePix error model in Rosetta Resolver version 5.0 (Rosetta Biosoftware, Seattle, WA, USA). Microarray data for the tumour and normal samples were compared using an error-weighted ANOVA model and corrected for multiple testing[19] in Rosetta Resolver. Microarray data are available on Gene Expression Omnibus with accession number GSE21181.

Bisulphite sequence analysis (BSA)

DNA samples (500 ng) were converted using the EZ DNA methylation Gold bisulphite kit (Zymo Research). Primers (Supplementary Table S3) for BSA of ten CpGs in the *PTPRGint1* locus (Supplementary Figure S1) were designed using MethPrimer.[20] Amplification was carried out in a DNA Engine Dyad Peltier Thermal Cycler (Bio-Rad, Hercules, CA, USA) using AmpliTaq Gold PCR buffer and enzyme (Applied Biosystems, Foster City, USA). For the direct BSA, PCR products were sequenced using the right primer, resulting in nine interpretable CpGs (CpG2-10). For additional clonal BSA, *PTPRGint1* PCR fragments were cloned into TOP10 E. coli bacteria using a TOPO TA cloning kit (Invitrogen, Carlsbad, CA, USA). Sequence alignment analysis was performed using ClustalW[21] and BioEdit.[22] CpG dinucleotides in the direct BSA were scored as being methylated when the ratio of the cytosine/thymine peaks was above 0.4. The BiQ analyzer software was used for visualization.[23]

MS-MLPA assay

Custom MS-MLPA probes (Supplementary Table S3) for the *PTPRGint1* locus were designed in Primer3[24] and included the HhaI site in CpG9 (see Figure 1B). As a control we used a *BRCA2* probe set from the SALSA MS-MLPA KIT ME001B Tumour suppressor-1 kit (MRC-Holland, Amsterdam, The Netherlands). Fragment analysis was performed on an ABI 3130 (Applied Biosystems, Foster City, US). The MS-MPLA was performed as described using 50 ng of genomic DNA[25]. A negative, unmethylated control (human semen DNA) and a 100% methylated DNA control (CpGenome Universal methylated DNA, Chemicon, Millipore, Billerica, MA, USA), were included in every experiment to asses HhaI cleavage and PCR. Fragment analysis was performed in GeneMapper (Applied Biosystems). *PTPRGint1* peak heights were normalized by comparison with the *BRCA2* peak height from the same run. Subsequently, the *PTPRGint1*/*BRCA2* ratio from the digested reaction was divided by the *PTPRGint1*/*BRCA2* ratio from the undigested reaction resulting in one ratio per sample that represented the fraction of methylated CpG9. Ten independent measurements provided a ratio distribution for unmethylated (mean 0.108, sd 0.037) and fully methylated (mean 0.833, sd 0.148) control DNA. Samples were typed as being unmethylated or methylated when they were within three standard deviations of the mean of the unmethylated and methylated reference samples, respectively. Samples with ratios in between these standard deviation boundaries were scored as partially methylated. For specificity and sensitivity calculations, partially methylated samples were considered methylated. Determination of specificity between normal and tumour tissue was performed by a Chi-square test.

Real-time RT-PCR

cDNA was generated using the random primer protocol from the Transcriptor First Strand cDNA Synthesis Kit (Roche Applied Science, Indianapolis, IN, USA) using 1 µg of RNA. Intron-spanning primers (Supplementary Table S3) were designed to target exon 1 and 2 of the main *PTPRG* transcript in Primer3[24]. Reactions were performed in duplicate on a Light Cycler 480 (Roche Applied Science) using IQ SYBR Green SuperMix (Biorad). High resolution melting curve analysis was performed to check primer specificity. Reactions with more than one peak in the melting curve were discarded, as were samples where the standard deviation between technical replicates was above one Ct value. A standard curve was generated using five 1:10 dilutions of pooled cDNA from colon cancer cell lines (SW48, RKO, SW480, Caco2, SW837, and LS411), showing an efficiency of 100%. The Ct values that were used for analysis were between 23 and 33. Relative mRNA concentrations were calculated from this standard curve. Stably expressed control genes for normalization were selected with the GeNorm applet; the two most stably expressed genes were used for normalization of each tumour cDNA (*CPSF6* and *EEF1*)[26].

CTCF ChIP and quantitative PCR

The primary colon cancer cell line KP7038t, established at the Department of Pathology at the LUMC, was grown in GIBCO RPMI 1640 with glutamax (Invitrogen, Carlsbad, CA, USA), 10% foetal calf serum, and penicillin/streptomycin (50µg/ml). Tumour-associated fibroblasts (KP7038f), collected from the primary tumour, as well as cell lines RKO and SW480 were grown under identical conditions. Cells at approximately 80% confluency were fixed with 1% formaldehyde for 10 minutes at room temperature. The formaldehyde was quenched with glycine (0.125 M) and the cells were harvested by scraping. Chromatin immunoprecipitations were performed using the SimpleChIP Enzymatic Chromatin IP kit (Cell Signaling Technology, Danvers, MA, US) with 10 µl anti-CTCF antibody (D31H2 XP; Cell Signaling Technology). Normal colon mucosa was collected from a patient who underwent a colon colonoscopy unrelated to colon cancer. Twenty 50µm sections, cut from macro-dissected frozen tissue, were fixed in PBS with 1% formaldehyde. After quenching, the tissue was micro-dissected and processed for ChIP followed by duplicate PCR reactions as above. Primers (Supplementary Table S3) targeting control regions were selected from Kim *et al*.[27]. The Ct values that were used for analysis were between 25 and 40. Standard curves were generated using four consecutive 1:5 dilutions of input DNA for both cultures (non-immunoprecipitated, cross-linked DNA) to determine relative DNA concentrations. For comparison between pull-downs, relative DNA concentrations of the CTCF and IgG antibody pull-downs were normalized with the corresponding relative concentration from the histone H3 antibody pull-down.

Results

Locus PTPRGint1 is methylated in colorectal adenomas and carcinomas

Methylation profile comparison by ANOVA of 15 carcinomas, three adenomas and eight paired normal mucosa samples identified 20 differentially methylated loci for the three tissue groups (false discovery rate < 0.01%). For all but one of these loci methylation in the adenomas was comparable to the normal samples while carcinomas showed increased methylation (Figure 1A). The most significant CpG island clone was 47B02 that showed increased methylation in both adenomas and carcinomas compared to normal mucosa. Therefore, we performed validation experiments for the corresponding locus, which mapped to the first intron of the *PTPRG* gene (chr3: 61524993-61525363, UCSC assembly: March 2006, see Supplementary Figure S1), referred to as *PTPRGint1*.

Analysis at single CpG resolution using direct BSA showed that 17 out of 18 tumour samples were fully methylated in the *PTPRGint1* region, while one carcinoma was unmethylated for CpG dinucleotides 8-10 (Figure 1B, bottom panel). In contrast, normal colon samples were mostly unmethylated (Figure 1B, upper panel). CpGs 7-10 were most informative to distinguish between tumour and normal in this set of samples. These results were confirmed at the single chromosome level using clonal BSA (Supplementary Figure S2). In summary, the microarray-based finding of differential *PTPRGint1* methylation in right-sided tumours was confirmed, and extended to left-sided adenomas and carcinomas.

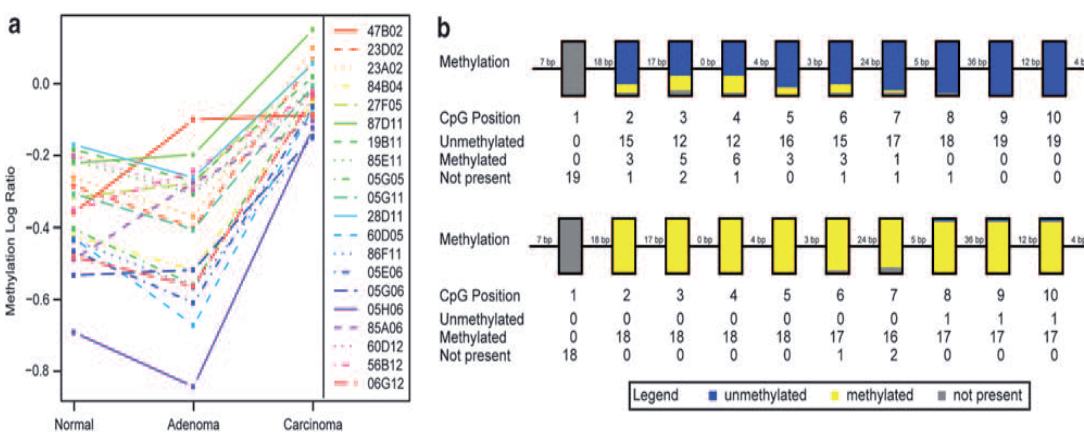


Figure 1- Identification and validation of the differentially methylated locus *PTPRGint1*. (A) Trend plot of the top-20 differentially methylated microarray clones (FDR ≤ 0.01%) showing the average log10 ratios in the normal, adenoma and carcinoma samples compared to the colorectal cancer cell line reference panel. Clone 47B02, corresponding to *PTPRGint1*, had a similarly increased log ratio in adenomas and carcinomas compared to normals (red solid line). (B) BiQ summary of direct BSA of *PTPRGint1* (CpG dinucleotides 1-10) in paired normal colon mucosa (n=19, upper panel) and colon tumours (n = 18, 12 right- and left-sided carcinomas and 6 adenomas, lower panel) showed highest specificity and sensitivity in the four most 3' CpGs measured. Each box corresponds to one CpG position in the genomic sequence, while colours summarize the methylation states of all sequenced samples at that position.

Methylation of PTPRGint1 CpG9 has high sensitivity and specificity

To assess the sensitivity and specificity of *PTPRGint1* methylation to discriminate between cancer and normal tissue, we developed a high-throughput MS-MLPA assay (Figure 2A, 2B). We tested an FFPE cohort of 103 tumours and 58 corresponding normal tissues, which allowed us to assess the possibility of age-related methylation often seen in aging mucosa. Of the 67 carcinoma samples 94% showed methylation of the targeted CpG dinucleotide (61 fully, two partially methylated), while 46 (95.8%) of the 48 corresponding normal samples were unmethylated, and the remaining two partially methylated (Figure 2C, Table 1). Comparing the methylation numbers between normal and tumour tissue by Chi-square test provided a highly significant p-value of $9.8x10^{-110}$. *PTPRGint1* methylation was independent of MSI status in the sporadic carcinomas, as both MSI-High and stable cases were methylated. A relatively small group of 18 sporadic adenomas (13 low grade dysplastic and five high grade dysplastic) and neighbouring normal mucosa of 10 of these showed *PTPRGint1* methylation in all adenomas, but not in the available normal mucosa (Figure 2C, Table 1). To assess whether *PTPRGint1* methylation is an early event in colorectal carcinogenesis, several colon lesions preceding the adenoma/carcinoma stages were studied. All six hyperplastic polyps and 11 out of 12 serrated adenomas tested showed *PTPRGint1* methylation (Figure 2C).

Table 1: Sensitivity and specificity of the *PTPRGint1* locus CpG9 methylation in sporadic tumours and tumours associated with a specific MMR mutation

CpG9	Sensitivity Methylated tumours / Total Tumours	Specificity [1] Unmethylated normals / Total normals
Sporadic adenomas	100% (18/18)	100% (10/10)
Sporadic carcinomas	94% (63/67)	95.8% (46/48)
MLH1 mutated	100% (14/14)	87% (20/23)
MSH2 mutated	96% (18/19)	100% (24/24)
MSH6 mutated	86.7% (26/30)	100% (14/14)
Total Lynch	92.1% (58/63)	95.4% (62/65)

[1]The mutational analysis of the MMR genes was incomplete for four unpaired normal samples in the Lynch syndrome cohort. Therefore, these were only included in the total specificity calculations.

PTPRGint1 CpG9 methylation in Lynch syndrome associated colorectal cancer

The initial cohort for MS-MLPA validation contained two Lynch syndrome associated colorectal carcinomas that were both methylated. Therefore, we further investigated *PTPRGint1* methylation in 63 carcinomas from patients with a DNA mismatch repair (MMR) gene mutation (14 *MLH1* mutations, 19 *MSH2* mutations and 30 *MSH6* mutations). Of these 92.1% showed methylation (58 fully, 3 partially methylated), while 62 of the 65 (95.7%) corresponding normal samples were unmethylated (Figure 2C, Table 1; Chi-square p-value $3.3x10^{-153}$). In conclusion, methylation of *PTPRGint1* CpG9 has similarly high sensitivity and specificity in Lynch syndrome associated colorectal carcinomas as in sporadic colorectal cancer.

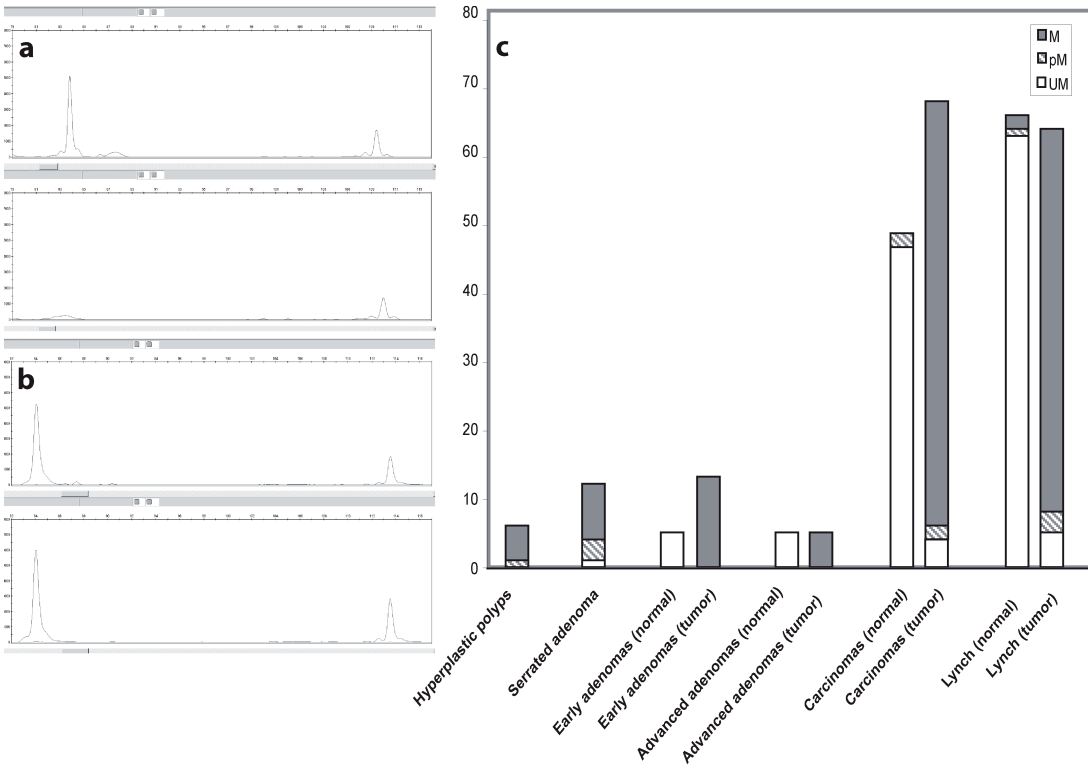


Figure 2 - *PTPRGint1* methylation detected by MS-MLPA. (a) GeneMapper output of the custom MS-MLPA to analyze *PTPRGint1* CpG9 methylation in a normal mucosa sample. Upper panel: The *PTPRGint1* peak was located at ~84 bp, the control peak was located at ~110 bp. Lower panel: Loss of *PTPRGint1* signal after HhaI digestion indicated an unmethylated CpG9. (b) GeneMapper output of the custom *PTPRGint1* MS-MLPA in the corresponding colon cancer sample. Upper panel: Undigested. Lower panel: HhaI digestion. Retention of the *PTPRGint1* marker signal indicated protection against HhaI digestion by CpG9 methylation. (c) Frequency of *PTPRGint1* CpG9 methylation in precursor lesions (hyperplastic polyps and serrated adenoma), early- and advanced adenomas, carcinomas, and corresponding normal mucosal tissue for the latter three groups. The number of samples typed as methylated (dark), partially methylated (striped) and unmethylated (white) in the MS-MLPA assay is indicated on the y-axis.

No silencing of PTPRG gene expression in methylated samples

To assess if hypermethylation of *PTPRGint1* affected expression of the gene, relative mRNA levels of *PTPRG* were studied in 15 right-sided colon carcinomas, three adenomas and 18 corresponding normal mucosa samples. The *PTPRG* gene encodes four protein coding isoforms (Source: HGNC Symbol; Acc:9671, aligned to Ensembl GRCh37). Using intron 1 spanning primers the full length transcripts ENST00000474889 and ENST00000295874 (missing one cassette exon) were analyzed (Figure 3). Two additional transcripts, ENST00000383711 and ENST00000394462 both starting at exon 3, were analyzed using intron 26 spanning primers and gave comparable results (data not shown). Expression of the *PTPRG* gene was detected in all samples. To assess the effects of *PTPRGint1* methylation on *PTPRG* expression, we compared the MS-MLPA methylation ratio with the *PTPRG* relative expression (Figure 3). We found that *PTPRG* was expressed at similar levels in the tumour and normal samples independent of methylation status. One patient (No. 28) showed increased *PTPRG* expression in both normal and tumour samples, thought to reflect individual expression differences and a possible copy number effect in the tumour.

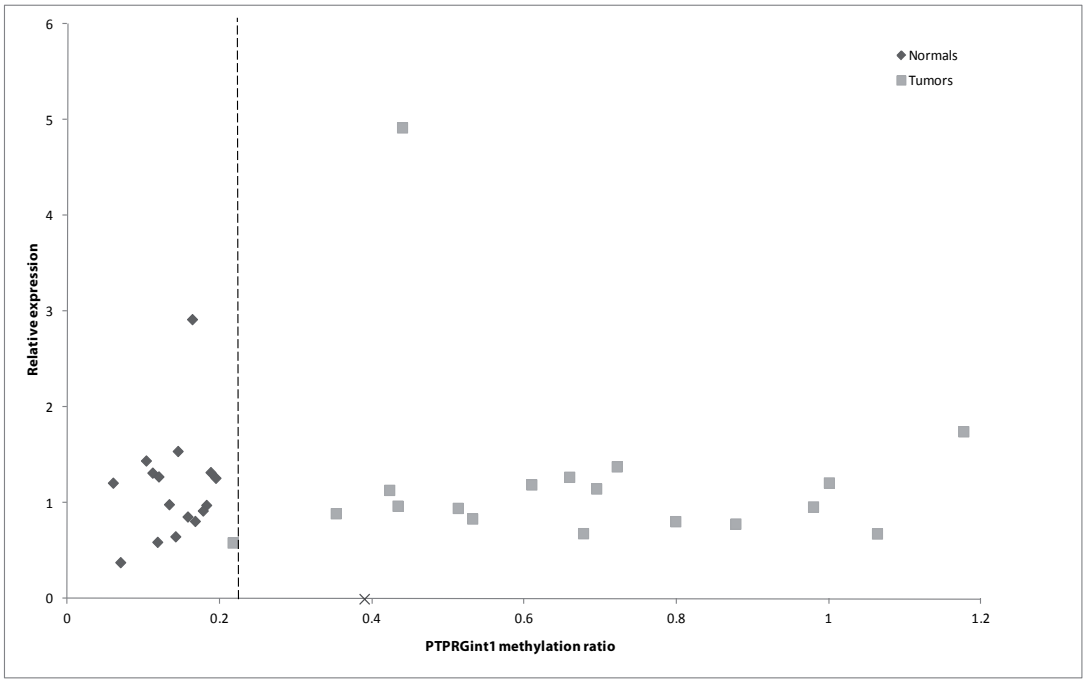
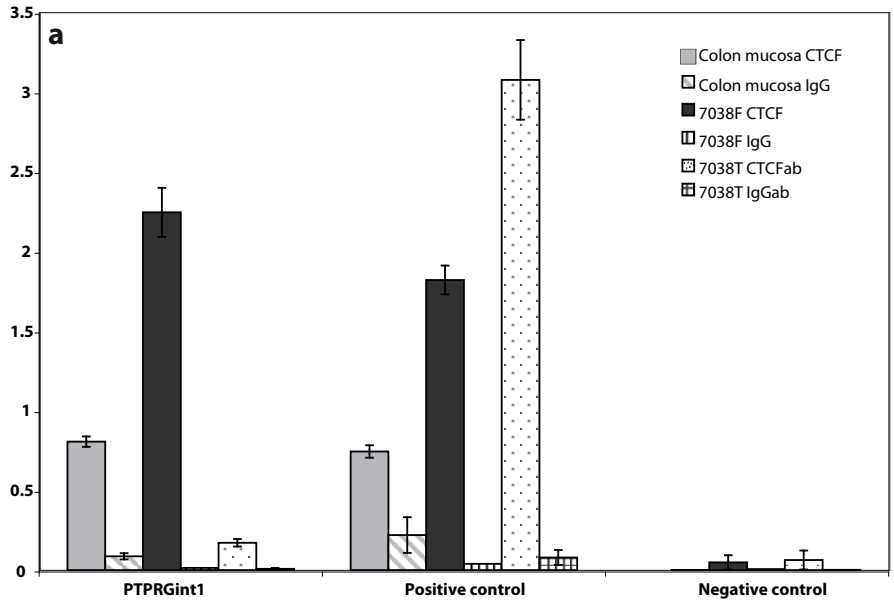


Figure 3 - Scatter plot of the relative *PTPRG* expression against the *PTPRGint1* methylation ratio according to the MS-MLPA. The dotted vertical line at 0.22 indicates the cut-off for unmethylated samples. Plotted data is representative of two independent experiments.

PTPRGint1 is a methylation-dependent CTCF binding site

The *PTPRGint1* locus overlapped with an experimentally defined CTCF binding site from a CTCF ChIP-chip study in fibroblasts[27] displayed in the genome web browsers USCS[28] and Ensembl[29]. This CTCF binding region of 750bp (OREG0015647; chr3: 61525101-61525851, UCSC assembly: March 2006) has a 262 bp overlap with the 3' part of the *PTPRGint1* locus (Supplementary Figure S1). We studied CTCF binding by ChIP in the primary tumour culture KP7038t that carried *PTPRGint1* methylation (MS-MLPA ratio 0.97, data not shown), unmethylated tumour-derived fibroblasts KP7038f from the same patient (MS-MLPA ratio 0.07, data not shown), as well as normal colon mucosa. We detected CTCF binding to *PTPRGint1* in the normal mucosa and KP7038f fibroblasts, but little binding to the primary tumour cells (Figure 4A).

To control for possible differences in the amount of CTCF protein between the samples, we compared the qPCR results for the *PTPRGint1* region with a positive control locus 7.9 Mb distant from *PTPRGint1* that was shown to bind CTCF and does not contain a CpG in its putative 20 bp consensus sequence[27]. This locus was enriched in pull-downs of all samples, including the primary tumour cells, indicating that lack of CTCF binding to *PTPRGint1* was not due to lack of CTCF protein. CTCF binding to *PTPRGint1* was comparable to the positive control in both KP7038f fibroblasts (ratio 1.2) and normal colon mucosa (ratio 1.1). However, the *PTPRGint1*/positive control ratio was 0.06 in the primary tumour culture KP7038t (Figure 4B). CTCF binding to *PTPRGint1* was similarly decreased in colorectal cancer cell lines RKO and SW480 (Figure 4B). These results indicate a significant decrease of bound CTCF to the methylated *PTPRGint1* region in tumour cells.



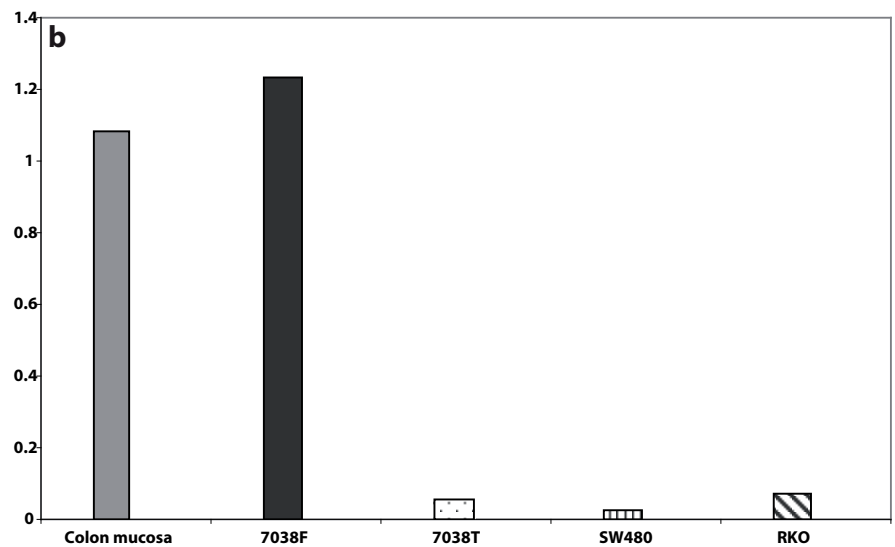


Figure 4 - (a) CTCF binding to *PTPRGint1*, positive- and negative control regions in normal colon mucosa (light grey), KP7038f (black) and KP7038t (dotted). The histone H3 normalized values of the CTCF antibody and IgG negative control antibody pull downs are given per primer pair. Standard errors represent the variability of duplicate PCR reactions. This is a representative experiment of three ChIPs. (b) The *PTPRGint1*/positive control ratio for normal colon mucosa, KP7038f, KP7038t (also shown in A), SW480 (vertically striped) and RKO (diagonally striped).

Discussion

We describe colorectal tumour-specific methylation of a locus in the first intron of the putative tumour suppressor gene *PTPRG* in both proximal and distal carcinomas and adenomas, including Lynch syndrome tumours. For these high-risk individuals, who are advised to undergo regular colonoscopies, no molecular markers have been described so far[30]. Assuming that a successful faecal or blood DNA test for *PTPRGint1* methylation could be developed, this is a promising discovery that would aid the early detection of colorectal tumours, independent of their aetiology.

PTPRGint1 is not located in a promoter CpG island but in the first intron, about 3kb from the transcriptional start site. We did not find a relation between the methylation status of *PTPRGint1* and *PTPRG* expression, indicating that *PTPRGint1* methylation does not lead to loss of function of PTPRG as has been observed for mutations in colon cancer, and deletions in lung carcinomas and renal carcinoma cell lines[31,32]. However, the identification of a methylation-sensitive CTCF binding site overlapping with *PTPRGint1* suggests that tumour-specific methylation may have a more distant effect. Differential binding of the insulator protein CTCF could have a major influence on expression of distant genes through alternative loop formation, as has been observed in β-globin expression in mouse models[33]. A recent study has shown that loss of CTCF binding to a boundary region upstream of *CDKN2A* resulted in spreading of repressed chromatin and DNA methylation into the p16 promoter with sequential down-regulation of *p16* expression[34]. The same study described that loss of upstream CTCF binding resulted in promoter DNA methylation of *RASSF1* and *CDH1*[34]. Contradictory to the finding that CTCF binding abrogation was shown to be causative of heterochromatin spreading and DNA methylation[34,35], is the observation that DNA methylation of CTCF binding sites is suggested to regulate CTCF binding[36, 37]. Interestingly, aberrant DNA methylation that excludes CTCF binding to intronic regulatory DNA was shown to promote expression of an oncogene, *BCL6*, in B cell lymphomas[38]. Although the sequence of events is unknown, the age-related aberrant hypermethylation often seen in colon cancer hints towards the latter.

We excluded differential peptidic abundance of CTCF between tumour and normal samples by successfully amplifying a positive control CTCF binding site on all samples. It remains to be demonstrated if CTCF protein modifications and its cellular and nuclear distribution are maintained in all tumour cells, both of which can influence CTCF activity and the binding to specific regions. More insight into the role of aberrant DNA methylation of *PTPRGint1* in the aetiology of cancer requires a better understanding of whether aberrant CTCF binding is caused by inhibition of protein activity or by initial aberrant methylation of the CTCF binding site.

Hypermethylation of the CpG island in the *PTPRG* gene promoter has been previously described in cutaneous T-cell lymphomas, melanoma cell lines, and gastric cancer[39-41]. Transcriptional down-regulation was shown to be associated with *PTPRG* promoter methylation in the cutaneous T-cell lymphomas study[39]. This study used a similar microarray for identification of differential methylation as the present study. We did not find differential methylation of the *PTPRG* promoter region between normal and colon tumour samples on the microarray. Moreover, BSA of colorectal cancer cell lines showed that the *PTPRG* promoter region was unmethylated (R. van Doorn, personal communication). Therefore, we have no indication for upstream spreading of DNA methylation from the CTCF binding

region at *PTPRGint1* towards the promoter.

In conclusion, this study provided evidence for tumour-specific hypermethylation of a CTCF binding site located in the first intron of *PTPRG*. The high specificity and sensitivity imply a possible utility for *PTPRGint1* methylation in new or existing colon-specific methylation marker panels. Especially the high level of *PTPRGint1* methylation in Lynch syndrome associated colorectal tumours is unique and could prove to be a valuable addition. Methylation-dependent absence of CTCF binding to the *PTPRGint1* locus suggests a possible effect on chromatin density or conformation that could play a role in colon tumorigenesis[42].

Acknowledgments

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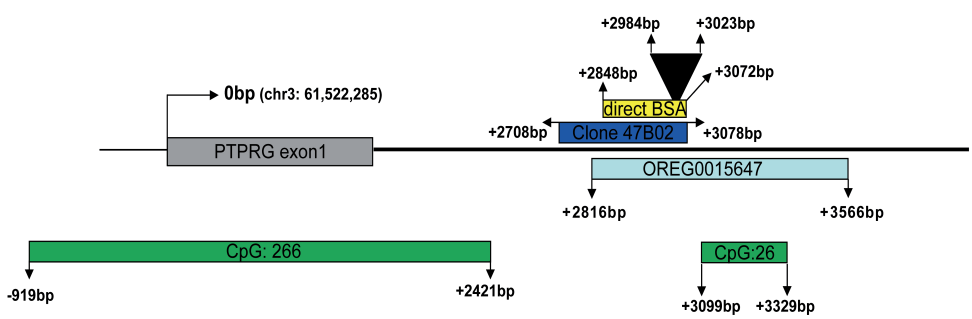
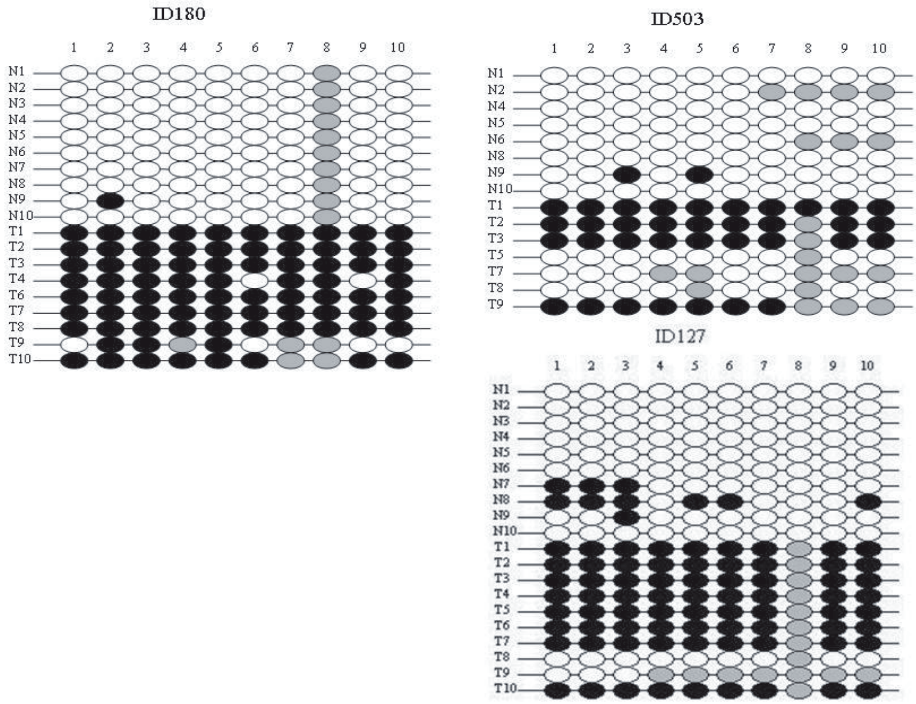
Conflict of interest

The authors (EHJvR, HM, and JB) have filed a patent application pertaining to intellectual property related in part to this report (P78692EP00 Submitted December 31, 2007). All other authors have nothing to disclose.

Supplementary data



Supplementary Figure S1 - Visual representation of the PTPRGint1 region. The *PTPRG* exon 1 (grey) and beginning of intron 1 (thick black line) are shown, with the transcription start site indicated with an arrow. Relative to the *PTPRG* start site, the following genomic features are shown: Promoter CpG island and intron 1 CpG island (green), ORegAnno CTCF fragment OREG0015647 (adapted from UCSC genome browser, light blue), CpG island microarray clone 47B02 (dark blue), amplified region by direct BSA (yellow). The black arrowhead indicates the approximate location of MS-MLPA probe hybridization. Visualization based on UCSC assembly: March 2006.



Supplementary Figure S2 - Clonal BSA of PTPRGint1 in three paired tumour-normal tissues**.** Visual representation of the clonal BSA results of ten CpG dinucleotides of PTPRGint1 in carcinomas ID503 and ID127 and adenoma ID180. Black dot: methylated CpG. White dot: unmethylated CpG. Grey dot: sequence not readable. In a partially methylated normal sample (ID127) not more than three out of ten alleles were found methylated for several CpGs. In addition, complete methylation of 9/9, 8/10 and 4/7 sequenced tumour alleles was found respectively, roughly corresponding to the estimated percentage of tumour cells observed in HE-stained tissue sections.

Supplementary Table S1 - Overview of the sporadic colorectal tumour samples and paired controls used in this study. Location, histology, age, sex and microsatellite instability status are listed, as well as the different techniques used on each sample. A gray box behind a sample in one of the last five columns indicates that the sample was tested with the technique listed in the header. Available at: http://www.nature.com/ejhg/journal/v19/n3/suppinfo/ejhg2010187s1.html?url=/ejhg/journal/v19/n3/full/ejhg2010187a.html

Supplementary Table S2 - Overview of Lynch cohort used. Location, histology, mismatch repair gene mutation, age and sex are listed for each sample as well as the different techniques used in this study. A gray box in the last column indicates that the sample was tested with the technique listed in the header. Available at: http://www.nature.com/ejhg/journal/v19/n3/suppinfo/ejhg2010187s1.html?url=/ejhg/journal/v19/n3/full/ejhg2010187a.html

Supplementary Table S3 Overview of the primers used in this study. Available at: http://www.nature.com/ejhg/journal/v19/n3/suppinfo/ejhg2010187s1.html?url=/ejhg/journal/v19/n3/full/ejhg2010187a.html

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