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The novel colorectal cancer biomarkers *CDO1, ZSCAN18* and *ZNF331* are frequently methylated across gastrointestinal cancers

Hege Marie Vedeld[1,2,3], Kim Andresen[1,2,4], Ina Andrassy Eilertsen[1,2], Arild Nesbakken[2,5,6], Raquel Seruca[7], Ivar P. Gladhaug[5,8], Espen Thiis-Evensen[9], Torleiv O. Rognum[5,10], Kirsten Muri Boberg[4,5,9] and Guro E. Lind[1,2,3]

[1] Department of Cancer Prevention, Institute for Cancer Research, Oslo University Hospital- Norwegian Radium Hospital, Oslo, Norway
[2] Centre for Cancer Biomedicine, Faculty of Medicine, University of Oslo, Oslo, Norway
[3] Department of Biosciences, University of Oslo, Oslo, Norway
[4] Norwegian PSC Research Center, Department of Transplantation Medicine, Oslo University Hospital, Oslo, Norway
[5] Faculty of Medicine, University of Oslo, Oslo, Norway
[6] Department of Gastrointestinal Surgery, Oslo University Hospital- Aker, Oslo, Norway
[7] Institute of Molecular Pathology and Immunology, University of Porto, Portugal
[8] Department of Hepato-Pancreato-Biliary Surgery, Oslo University Hospital, Rikshospitalet, Oslo, Norway
[9] Section of Gastroenterology, Division of Cancer, Surgery and Transplantation, Department of Transplantation Medicine, Oslo University Hospital, Oslo, Norway
[10] Department of Forensic Pathology and clinical Forensic Medicine, Norwegian Institute of Public Health, Oslo, Norway

We have previously shown that gastrointestinal cancers display similar epigenetic aberrations. In a recent study, we identified frequently methylated genes for cholangiocarcinoma (*CDO1*, *DCLK1*, *SFRP1* and *ZSCAN18*), where one of these genes, *DCLK1*, was also confirmed to be highly methylated in colorectal cancer. The aim of the present study was to determine whether these four genes, in addition to one gene found to be methylated in colon cancer cell lines *(ZNF331)*, are commonly methylated across gastrointestinal malignancies, as well as explore their role as potential biomarkers. Quantitative methylation specific PCR (qMSP) of colorectal cancer ($n = 164$) and normal colorectal mucosa ($n = 106$) samples showed that all genes were frequently methylated in colorectal cancer (71–92%) with little or no methylation in normal mucosa (0–3%). Methylation of minimum two of these five genes identified 95% of the tumors with a specificity of 98%, and an area under the receiver operating characteristics curve (AUC) of 0.98. For gastric ($n = 25$) and pancreatic ($n = 20$) cancer, the same panel detected 92% and 90% of the tumors, respectively. Seventy-four cancer cell lines were further analyzed by qMSP and real time RT-PCR. In addition to the previously reported *DCLK1*, a high negative correlation between promoter DNA methylation and gene expression was observed for *CDO1*, *ZNF331* and *ZSCAN18*. In conclusion, the high methylation frequency of these genes in colorectal- as well as in gastric-, pancreatic- and bile duct cancer confirmed an epigenetic similarity between gastrointestinal cancer types, and simultaneously demonstrated their potential as biomarkers, particularly for colorectal cancer detection.

Early Detection and Diagnosis

Key words: biomarker, cancer, *CDO1*, *DCLK1*, DNA methylation, *SFRP1*, *ZNF331*, ZSCAN18

Abbreviations: AUC: area under the ROC curve; AZA: 5-aza-2′deoxycytidine; PMR: percent methylated reference; qMSP: quantitative methylation specific PCR; ROC: receiver operating characteristics; RT-PCR: reverse transcription PCR; TSA: trichostatin-A

Additional Supporting Information may be found in the online version of this article.

Disclosure: A US provisional patent application has been filed describing methylation of CDO1, DCLK1, ZNF331, and ZSCAN18 as biomarkers for detection of gastrointestinal cancers (61/451,198, INVEN-31899/US-1/PRO). Andresen and Lind are two of the inventors.

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Correspondence to: Guro E. Lind, Department of Cancer Prevention, Institute for Cancer Research, Oslo University Hospital, The Norwegian Radium Hospital, Montebello, 0310 Oslo, Norway, Tel.: +47-22781729, Fax: +47-22935767, E-mail: guro.elisabeth.lind@rr-research.no

What's new?

Various types of gastrointestinal (GI) cancers display similar epigenetic aberrations. In this study, the authors examined a number of genes that have been shown to have altered methylation in cholangiocarcinoma, to see whether these genes might also be altered in other GI cancers. They found five genes that are frequently methylated in colorectal, pancreatic, and gastric cancer and cholangiocarcinoma. Methylation patterns in these genes may therefore provide biomarkers that are especially promising for colorectal cancer detection, with a high combined sensitivity (95%) and specificity (98%).

Gastrointestinal cancers include malignancies arising in the esophagus, liver and bile ducts, gallbladder, pancreas, stomach, small intestine, colon and rectum. Their incidence and mortality differ significantly, however, together they account for approximately one-fifth of the cancer incidence and nearly one-fourth of the cancer related deaths in the US.[1] Colorectal and gastric cancers are the most common gastrointestinal tumors worldwide, accounting for ~2.2 million new cases and an estimated 1.3 million deaths annually.[2] When these malignancies are detected at an early, localized stage, the 5-year survival is 90% and 63%, respectively.[3] However, more than 60% of the patients with colorectal cancer and 75% of the patients with gastric cancer have regional or distant metastases at the time of diagnosis, resulting in a significant drop in survival.[3] Cholangiocarinoma and pancreatic cancer are less prevalent.[2,4] However, these malignancies are often "clinically silent" and thus diagnosed at an advanced stage with a corresponding poor prognosis.[2,4] Early detection, particularly of colorectal and gastric cancer, may significantly reduce the number of gastrointestinal cancer deaths, and identification of suitable biomarkers for this purpose is therefore warranted.

In humans, DNA methylation occurs predominantly at the 5′ position of cytosine, within CpG dinucleotides. Approximately 70% of the human genes have a CpG island—an enrichment of such dinucleotides—in the promoter region.[5] In normal cells these islands are usually unmethylated, but several of them become hypermethylated in cancer and this is associated with repressed or lost gene expression.[6] Using genome-wide analyzes, DNA methylation was recently shown to be more frequent than genetic changes in colorectal cancer.[7] In addition to being frequent, aberrant DNA methylation has been shown to be an early event in tumorigenesis.[8] Finally, several examples of genes with promoter DNA hypermethylation have been detected in various bodily fluids from cancer patients, including bile, feces, plasma and urine,[9–13] indicating that methylation biomarkers may be useful for non- or minimally invasive cancer diagnostics.

Although cancer is a highly heterogeneous disease, comprising more than 200 different conditions, it is believed that tumors arising in the gastrointestinal tract share several molecular alterations. Gut derived adenocarcinomas have indeed been found to display similar copy-number changes, forming tissue-specific clusters when various cancer types were clustered according to amplification activated oncogenes.[14,15] Gastrointestinal tumors further show extremely high frequencies of transition mutations at CpG dinucleotides,[16] and several genes, including *APC*, *MLH1*, *MGMT*, *p16^{INK4a}*, *CDH1*, *SFRP1*, *RASSF1A* and *VIM* have been shown to be epigenetically dysregulated across malignancies of the gastrointestinal tract.[17–21] With increased focus on cross-tumor analysis, exemplified by The Cancer Genome Atlas (TCGA) Pan-Cancer project, more shared abnormalities among the gastrointestinal cancers are expected to be uncovered.

In the present study we have investigated whether the recently identified methylation biomarkers for cholangiocarcinoma (*CDO1*, *DCLK1*, *ZSCAN18* and *SFRP1*)[22] were methylated also in other malignancies of the gastrointestinal tract. The promoter methylation frequency of these genes, in addition to *ZNF331*, previously shown to be methylated across several gastrointestinal cancer cell lines,[22] was analyzed in tissue samples from colorectal-, gastric- and pancreatic cancer patients.

Material and Methods

Cancer tissue samples and controls

Colorectal cancer. A total of 316 colorectal tissue samples were available for the quantitative methylation specific PCR (qMSP). The test set comprised 59 primary cancer (patient median age 71; range 33–92 years) and 50 normal mucosa samples (47 individuals; median age 55; range 22–86 years), obtained from several different hospitals in the south-east region of Norway in the period 1987–1989, and from deceased colorectal cancer free individuals (Institute of Forensic Medicine, University of Oslo), respectively. The validation set included 105 carcinomas (patient median age 71; range 29–93 years) from patients undergoing surgical resection at the Oslo University Hospital, Aker (OUH) from 2005 to 2007, and 56 normal mucosa samples (median age 67; range 63–72 years) obtained from a population-based sigmoidoscopy screening study (Telemark, Norway[23]). In addition, from 46 of the colorectal cancer patients operated at OUH normal mucosa was available for qMSP analysis. These samples had been taken as far away from the tumor as possible.

Previously published Affymetrix exon array data, including 125 colorectal cancer samples and 15 samples from the normal mucosa of cancer patients[24–26] (accession numbers GSE24550 and GSE29638; GEO) were reanalyzed in the present study for expression of the candidate genes. All samples were obtained from OUH and 97 of the cancers and 14 of the normal samples overlapped with the samples included in the qMSP analysis. Thirteen of the samples analyzed for exon

expression were matched cancer and normal. All colorectal samples were fresh frozen.

Cholangiocarcinoma. The cholangiocarcinomas and the non-malignant controls included in the present study have been described previously.[22] In brief, 13 fresh frozen and 26 formalin fixed and paraffin embedded (FFPE) cholangiocarcinomas were included along with 54 non-malignant control samples (fresh frozen, $n = 21$; FFPE, $n = 33$).

Pancreatic and gastric cancer. The 20 pancreatic cancer samples included in the study were archival material (FFPE) sourced from Oslo University Hospital, Rikshospitalet, Norway. The 25 gastric cancer samples were fresh frozen and collected at H. S. Joao, Porto, Portugal, and processed at the Institute of Molecular Pathology and Immunology, University of Porto, Portugal, in accordance to a protocol shared by both institutions.

Ethics

The research biobanks for colorectal cancers have been registered according to national legislation (numbers 2781 and 236-2005-16141). The study is part of a project approved by the Regional Committee (REC) for Medical and Health Research Ethics (numbers 1.2005.1629 and S-09282c 2009/4958). The research biobank for pancreatic adenocarcinoma has also been approved by REC (number S-05081, REK 2.2005.145). The gastric cancer samples were collected and made available to research after the approval of the ethics committee of H. S. Joao in compliance with the Helsinki Declaration (http://www.wma.net/e/policy/b3.htm).

Cancer cell lines

Seventy-four cell lines from 15 different cancer types were analyzed in the present study, including bile duct ($n = 6$), urinary bladder ($n = 4$), breast ($n = 8$), colon ($n = 19$), gall bladder ($n = 2$), gastric ($n = 4$), kidney ($n = 4$), leukemia ($n = 3$), lung ($n = 4$), malignant peripheral nerve sheath tumor (MPNST) ($n = 1$), ovarian ($n = 4$), pancreatic ($n = 6$), prostate ($n = 1$), testis ($n = 4$) and uterus ($n = 4$). Four of the colon cancer cell lines (HCT15, HT29, SW480 and SW48) were, in parallel with standard culturing, also treated with a combination of the epigenetic drugs 5-aza-2′deoxycytidine (AZA; 1 mM for 72 hr; Sigma-Aldrich, St Louis, MO), and trichostatin A (TSA; 0.5 mM added the last 12 hr; Sigma-Aldrich). All cell lines were routinely fingerprinted using the AmpFLSTR Identifiler PCR Amplification Kit (Life Technologies, (Applied Biosystems), Carlsbad, CA), and all commercially available cell lines were authenticated. Short tandem repeat (STR) results from the non-commercial cancer cell lines will be provided upon request.

Selection of candidate genes for analysis

We have previously identified *CDO1*, *DCLK1*, *SFRP1* and *ZSCAN18* to be frequently methylated in cholangiocarcinoma.[22] Interestingly, preliminary data indicated that these genes, in addition to *ZNF331*, displayed frequent promoter methylation also in cell lines from various gastrointestinal malignancies.[22] Subsequently, in this study all five genes were investigated for their promoter methylation status in tissue samples from colorectal cancer patients (test and validation sets) and cancer free controls, as well as in gastric and pancreatic cancers. Of note, we recently reported *DCLK1* to be frequently methylated in colorectal cancer.[27] Here we have included *DCLK1* to evaluate the methylation status of this gene also in gastric and pancreatic cancer.

DNA extraction and bisulfite treatment

DNA from the cancer cell lines and from the colorectal and gastric tissue samples was isolated using either a standard phenol/chloroform extraction protocol or magnetic beads. DNA from the formalin-fixed paraffin embedded pancreatic cancers was extracted using the QIAmp DNA kit (Qiagen, Qiagen Inc., Valencia, CA), and the ND-1000 Nanodrop (NanoDrop Technologies, Wilmington, DE) was used to measure DNA quantity and quality. DNA (1.3 μg) was bisulfite treated using the EpiTect Bisulfite Kit (Qiagen) according to the manufacturer's protocol. Following the conversion reaction, which was performed in an MJ Mini Personal Thermal Cycler (Bio-Rad, Hercules, CA), bisulfite converted DNA was purified using the QIAcube (Qiagen) automated pipetting system, and eluted in 40 μl elution buffer.

Direct bisulfite sequencing

To guide the design of qMSP primers and probe for *ZNF331*, the gene promoter was subjected to direct bisulfite sequencing in 14 cancer cell lines (colon, $n = 6$; cholangiocarcinoma, $n = 6$; gallbladder, $n = 2$). Primers were designed using Methyl Primer Express 1.0 (Life Technologies), and the fragment covered by the primers (forward primer: 5′-TTTTTGG GGTATGGTTTTATTA-3′; reverse primer: 5′-TCCTCATTA AACTATACCCCAA-3′) was located −238 to +121 relative to the transcription start site (NM_018555, hg19). The direct bisulfite sequencing procedure has been described previously.[28] Briefly, fragments were amplified for 35 cycles using HotStarTaq (Qiagen) and the purified samples were sequenced using the dGTP BigDye Terminator Cycle Sequencing Ready Reaction kit in an AB Prism 3730 sequencer (Life Technologies). The electroferograms were analyzed manually using Sequencing Analysis 5.2 (Life Technologies). The approximate amount of methylation at each CpG site was calculated by dividing the peak height of the cytosine signal with the peak height of the cytosine plus the thymine signal,[29] giving values ranging from 0 to 1. CpG sites with methylation frequencies in the range of 0–0.2 were considered unmethylated, 0.21–0.8 partially methylated, and 0.81–1 fully methylated. The methylation status of individual CpG sites determined by bisulfite sequencing of parts of the *ZNF331* promoter is illustrated in Supporting Information Figure S1.

Quantitative methylation specific PCR

qMSP assays for *CDO1*, *DCLK1*, *ZCSAN18*, *SFRP1* and *ALU* (control) were available from a previous study.[22] For *ZNF331*, the Primer Express Software 3.0 (Life Technologies)

was used to design primers and a TaqMan Minor groove binder (MGB) probe. All primer and probe sequences are listed in Supporting Information Table S1. The primers and probes were purchased from BioNordica (BioNordica, Medprobe, Oslo, Norway), and Life Technologies, respectively.

The qMSP reactions were carried out as previously described[30] in a 7900HT Real-Time PCR System (Life Technologies). In accordance with the recommendations from Life Technologies, fresh frozen samples (colon and gastric) amplified after cycle 35 were censored. For the FFPE samples (pancreas and cholangiocarcinoma), where the DNA was expected to be of reduced quality, cycle 40 was used as a censoring threshold. The median quantity value of triplicates was used for data analysis, and the ALU-C4 element was used to normalize for DNA input.[31] The qMSP results were calculated as percent of methylated reference (PMR) by dividing the normalized quantity of the samples by the normalized quantity of the positive control (IVD) and multiply by 100.

To ensure high specificity for each qMSP assay, the thresholds for scoring the colon cancer samples as methylated were set according to the highest PMR value across the test series of normal mucosa samples (Supporting Information Table S2). No normal samples were available from the gastric epithelium or the pancreas. Hence, for the fresh frozen gastric cancer samples, the scoring thresholds generated from the colorectal mucosa samples were used. For dichotomizing the archival pancreatic cancers into unmethylated and methylated groups, the scoring thresholds from an archival series of non-cancerous liver samples (published in Ref. 22) were used (Supporting Information Table S2). Samples with PMR values equal to or above the scoring threshold were considered to be methylated.

cDNA synthesis and real-time quantitative gene expression analysis

Total RNA (2 μg) was converted to cDNA using the High Capacity cDNA Reverse Transcription Kit (Life Technologies) and an MJ Mini Personal Thermal Cycler (Bio-Rad) according to the manufacturer's protocol.

The real time RT-PCR reactions were performed as previously described,[30] and included 1× one of the following commercially available TaqMan gene expression assays; *CDO1* (Hs00156447_m1), *ZNF331* (Hs00367929_m1), *ZSCAN18* (Hs00225073_m1), *VDAC2* (Hs00748551_s1; control) and *PES1* (Hs00362795_g1; control; Life Technologies), 1× TaqMan Universal Mastermix with UNG (Life Technologies) and 10 ng of cDNA in a final reaction volume of 20 μl. Samples were amplified in triplicates in 384 plates using a 7900HT Real-Time PCR System (Life Technologies). The relative expression levels were calculated by dividing the median quantity of the sample by the average median quantity of the two controls *VDAC2* and *PES1*.

Statistics

All statistical analyses were carried out using PASW 18.0 (SPSS, Chicago, IL) and GraphPad Prism 6 (GraphPad Software, Inc, San Diego, CA). Mann–Whitney *U* test was used to compare the PMR values of the candidate genes in the different tissues. Fisher's exact test and Spearman correlation analysis were used for analyzing categorical variables, while receiver operating characteristics (ROC) curve analyses were used to evaluate the performance of the methylation biomarkers. To measure whether there was any correlation between the PMR values (methylation) of *CDO1*, *ZNF331* and *ZSCAN18* and their expression in cancer cell lines and in cancer tissue samples, a Spearman and Pearson correlation analysis were performed. An independent sample *t*-test was applied to compare the expression of the candidate genes in cancer versus normal tissue samples. All *p*-values were derived from two-sided tests, and $p \leq 0.05$ was considered statistically significant.

Results

DNA methylation of colorectal cancers and controls

Across the test and validation sets, methylation of *CDO1*, *SFRP1*, *ZNF331* and *ZSCAN18* was observed in 92%, 92%, 71% and 74% of the colorectal cancer samples, respectively, with minimal methylation in the normal mucosa samples (*CDO1*, *ZNF331* and *ZSCAN18*, 2%; *SFRP1*, 3%). Individual methylation frequencies for the test and validation sets are listed in Supporting Information Table S3, and the distribution of PMR values are illustrated in Figure 1. When including the previously reported qMSP results for *DCLK1*,[27] methylation of at least two of the five genes was observed in 156 of the 164 colorectal cancers analyzed (95% sensitivity), and in only 2 out of the 106 normal mucosa samples (98% specificity; Fig. 2). The area under the ROC curve (AUC) for each gene ranged from 0.82 (*ZSCAN18*) to 0.96 (*CDO1*), with a combined AUC (the sum of PMR values) for the five markers of 0.98 (Fig. 3). For the 46 normal mucosa samples collected from the resection margin of cancer patients, promoter methylation of *ZNF331* and *ZSCAN18* was detected in only one sample (data not shown). Interestingly, *CDO1* was methylation positive in 65% of these samples (data not shown), suggestive of an epigenetic field defect. However, the methylation levels were significantly higher in the cancer samples compared to the normal cancer samples ($p < 0.001$).

When comparing methylation status with clinicopathological features and microsatellite instability (MSI) status, significant associations were observed between promoter methylation and *BRAF* exon 15 mutation for *DCLK1* ($p = 0.031$), *ZNF331* ($p < 0.001$) and *ZSCAN18* ($p = 0.036$), and between methylation and MSI for *ZNF331* and *ZSCAN18* ($p = 0.002$ and $p < 0.001$, respectively). Methylation of all the analyzed genes was additionally significantly more common in tumors located in the colon compared to those in the rectum ($p < 0.05$). No significant association with DNA methylation was seen for stage, and for the combined markers, no significant associations between clinicopathological features and MSI status were observed, except for tumor localization ($p = 0.005$). Results are

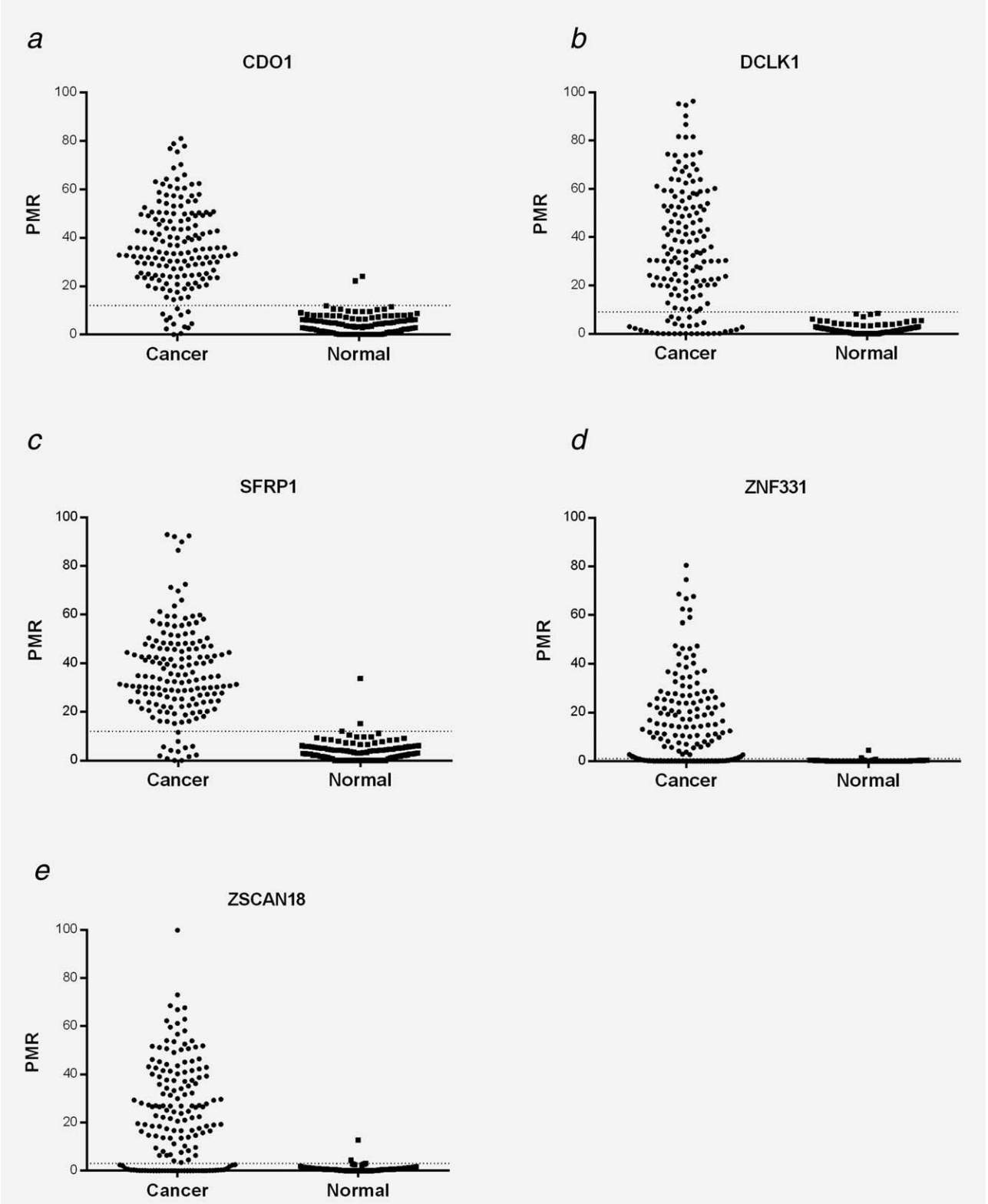

Figure 1. Promoter DNA methylation of candidate genes (*a–e*) in colorectal cancers and controls across test and validation sets. The scoring threshold is marked by a dotted line, and outliers are excluded for better visualization (*DCLK1*, $n = 6$; *SFRP1* and *ZSCAN18*, $n = 1$). Abbreviations; PMR, percent of methylated reference. The results for *DCLK1* have been published previously.[27]

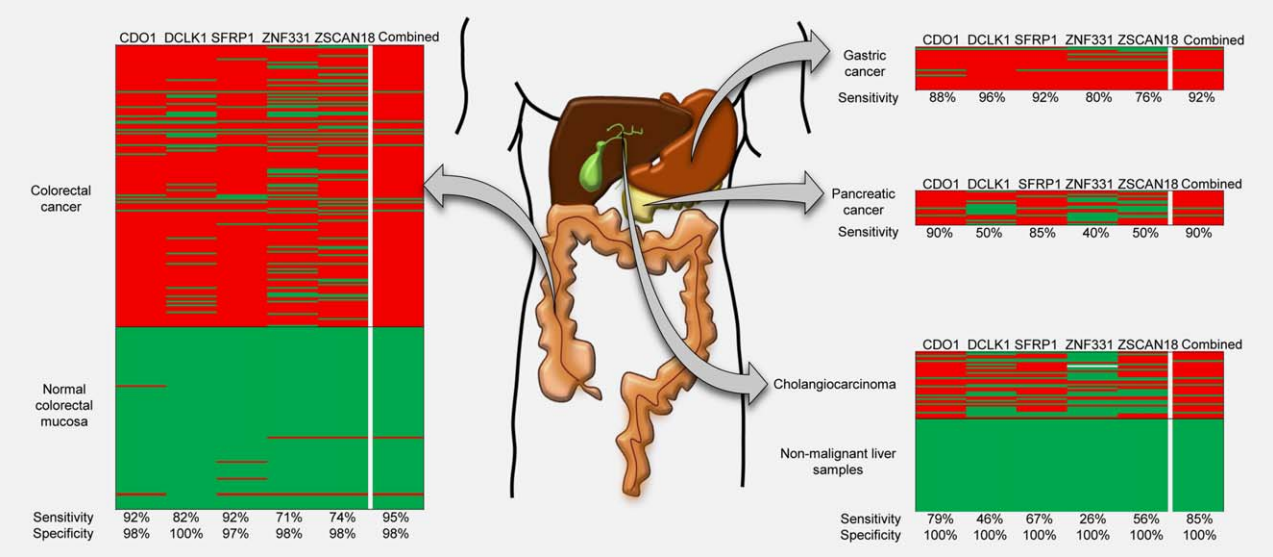

Figure 2. Promoter methylation frequencies of the candidate genes in gastrointestinal cancers and controls. For the "combined" column, a sample is considered methylation positive if a minimum of two of the five genes are methylated. Red: methylated; green: unmethylated; white: missing value. The methylation status for *DCLK1* in colorectal cancer and normal samples, as well as the cholangiocarcinoma results for *CDO1*, *DCLK1*, *SFRP1* and *ZSCAN18*, have been published previously.[22,27] The results are included here to evaluate the performance across gastrointestinal cancer types.

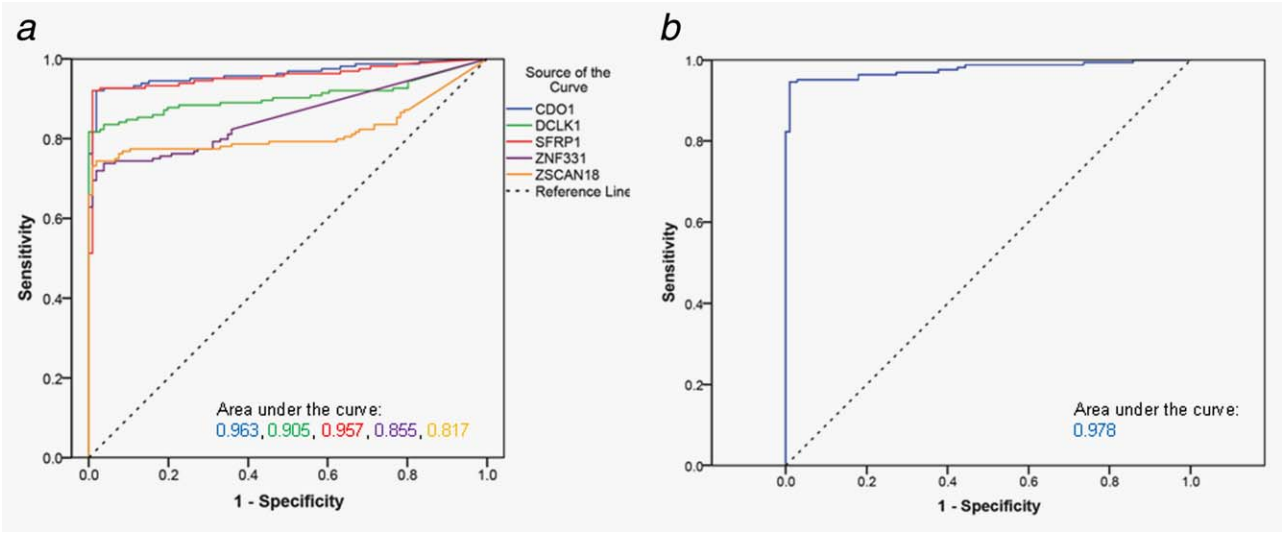

Figure 3. Receiver operating characteristic (ROC) curves for the markers individually (*a*) and combined (*b*) in colorectal cancer versus controls. The results for *DCLK1* have been published previously.[27]

summarized in Supporting Information Table S4. The median age of the donors of normal control mucosa samples in the test (55 years) and validation (67 years) sets was somewhat lower than that of the colorectal cancer patients (71 years). To rule out potential age-related bias contributing to the hypermethylation seen in the cancer patients, colorectal cancer samples were age matched with the normal controls. The gene promoter methylation frequencies of this subset of cancers were highly similar to the frequencies obtained across all the analyzed colorectal cancers (data not shown).

Gene expression analysis of CDO1, ZNF331 and ZSCAN18

Analyzing Affymetrix exon array data, *ZNF331* and *ZSCAN18* were significantly downregulated in 125 colorectal cancers compared to 15 normal mucosa samples (*ZNF331*, fold change 0.2, $p = 0.012$; *ZSCAN18*, fold change 0.2, $p = 0.005$). By combining the gene expression data with promoter methylation status (PMR values; qMSP), a negative correlation was further observed in colorectal cancer tissue samples ($n = 97$) for *ZNF331* (Pearson $r = -0.223$, $p = 0.028$) and *ZSCAN18* (Pearson $r = -0.430$, $p = 1.083 \times 10^{-5}$). No significant correlation

Table 1. Correlation between DNA methylation and expression of relevant target genes

Target gene	CDO1	ZNF331	ZCSAN18
RT-PCR assay ID	Hs0015644_m1	Hs00367929_m1	Hs00225073_m1
Spearman *r*	−0.482	−0.749	−0.810
95% confidence interval	[−0.644, −0.279]	[−0.837,−0.624]	[−0.878, −0.711]
P value (two-tailed)	<0.001	<0.001	<0.001

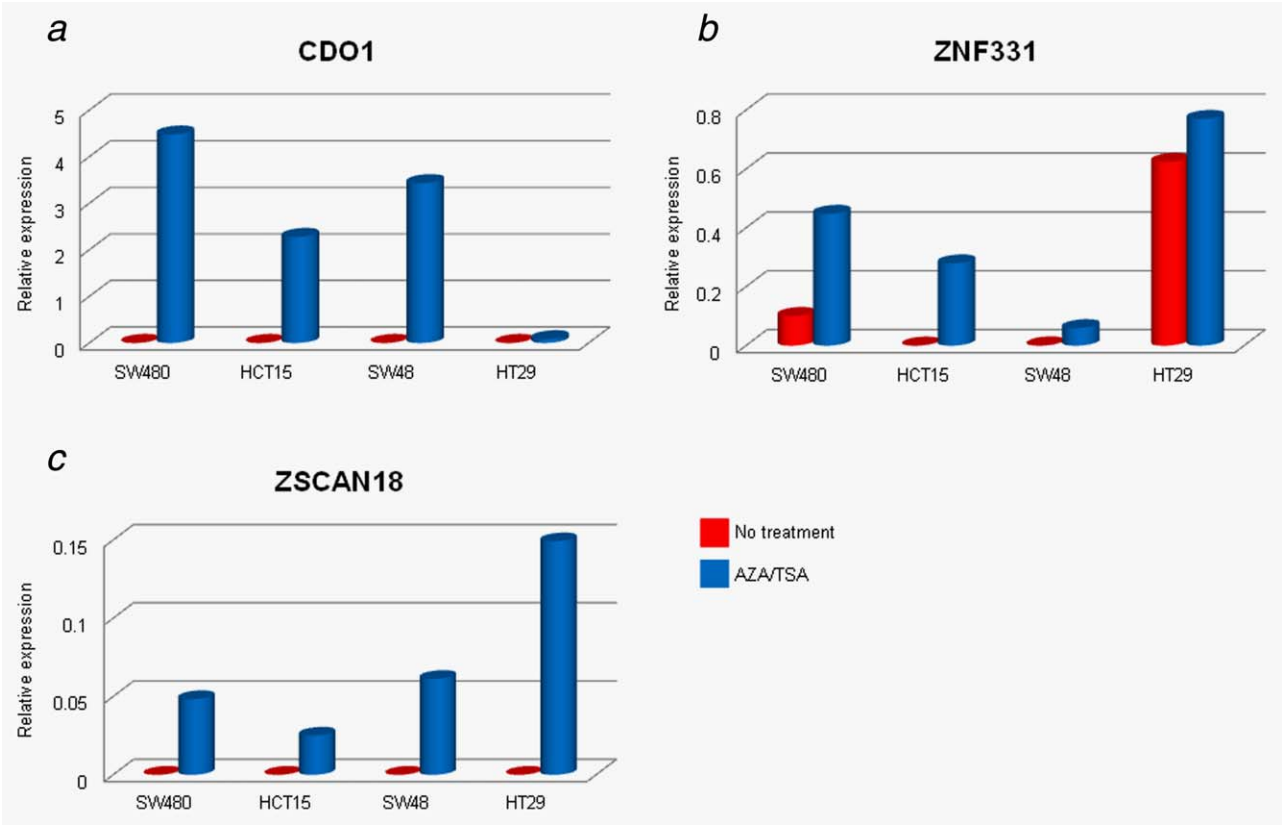

Figure 4. Expression of *CDO1*, *ZNF331* and *ZSCAN18* in four colon cancer cell lines before and after treatment with epigenetic drugs.

was observed between expression and methylation status for *CDO1*. Of note, only 11 of the 97 colorectal cancer samples were unmethylated, and when dividing the tumors into two groups based on location (colon and rectum) a significant negative correlation between expression and PMR values for *CDO1* was observed for rectal samples (Pearson $r = -0.442$, $p = 0.039$; methylated samples, $n = 16$; unmethylated samples, $n = 6$). Furthermore, a significant negative correlation between promoter methylation status (PMR values; qMSP) and the expression (real time RT-PCR) of all three genes was confirmed across 74 cancer cell lines derived from 15 different tissues (Table 1). Associations between expression and methylation have been reported previously for *DCLK1*[27] and *SPFRP1*,[32] and were therefore not included here.

Finally, the impact of the promoter hypermethylation on the gene expression was confirmed by real time RT-PCR in four colon cancer cell lines (HCT15, HT29, SW480 and SW48) before and after treatment with epigenetic drugs (AZA and TSA). Increased expression after epigenetic drug treatment was observed for all the analyzed genes (Fig. 4).

DNA methylation of gastric, pancreatic and bile duct cancer

Promoter methylation of *CDO1*, *DCLK1*, *SFRP1*, *ZNF331* and *ZSCAN18* was detected in 88%, 96%, 92%, 80% and 76% of the gastric cancer samples, respectively, and in 90%, 50%, 85%, 40% and 50% of the pancreatic cancer samples. Methylation of at least two of these five genes was seen in 23 of the 25 gastric cancers and in 18 of the 20 pancreatic cancer samples, giving sensitivities of 92% and 90%, respectively. Results are summarized in Figure 2. Previously reported results for cholangiocarcinoma are also included in the figure.[22] However, these data have been reanalyzed using a higher cycle threshold for the formalin-fixed paraffin embedded tissues

(40 instead of 35), and the results therefore deviate somewhat from the original publication. The qMSP results for *ZNF331* are novel (also for cholangiocarcinomas). Despite the high sensitivity of the five markers (*CDO1*, *DCLK1*, *SFRP1*, *ZNF331* and *ZSCAN18*; 85–95%) across the various cancer types, variations in both methylation frequency as well as PMR values were seen for the individual genes, and were in general significantly higher in colorectal and gastric cancer compared to cholangiocarcinoma and pancreatic cancer (Supporting Information Table S5).

Discussion

By analyzing biomarkers originally identified for cholangiocarcinoma in colorectal, gastric and pancreatic cancer, we have demonstrated that *CDO1*, *DCLK1*, *SFRP1*, *ZNF331* and *ZSCAN18* are frequently methylated across gastrointestinal malignancies. The markers were particularly promising for detection of colorectal cancer with a combined sensitivity of 95% and a specificity of 98% in tissue samples.

Although several molecular alterations are shared among gastrointestinal tumors, differences have also been reported. Tumors arising in the liver, pancreas and stomach frequently display *APC* promoter hypermethylation, whereas APC is more often silenced by gene mutation in sporadic colorectal cancer.[17,33] Chromosomal instability has also been shown to increase from lower to upper gut adenocarcinomas.[34] So far, only a limited number of aberrations have been analyzed across several gastrointestinal cancer types within the same study.[20,21,35–37] However, large-scale analyses of molecular aberrations across thousands of tumors, including several gastrointestinal carcinomas, are currently being generated by TCGA (http://cancergenome.nih.gov/). And with initiatives such as the Pan Cancer group, which focuses on identifying similarities and differences across cancers profiled by the TCGA Network, the number of papers reporting on cross-tumor findings are expected to increase rapidly. Although the biomarkers described here were methylated across the vast majority of the analyzed tumors, the individual methylation frequencies and PMR values varied somewhat between the various gastrointestinal cancer types. In general, colorectal and gastric cancers displayed higher PMR values compared to cholangiocarcinoma and pancreatic cancer. This could be related to the quality of the tissue samples. DNA from colorectal and gastric cancers was from fresh frozen tissues, whereas the DNA from pancreatic tumors and partly cholangiocarcinomas was derived from FFPE tissue, which represents a limitation to the comparison between gastrointestinal tumors in the present study. Although the differences were not statistically significant, we have previously shown that the methylation frequency of gene promoters in FFPE cholangiocarcinomas is generally lower than among fresh frozen cholangiocarcinomas.[22] Due to protein crosslinking and fragmentation, the DNA fragment lengths from FFPE tissues are usually shorter than those from fresh frozen material, and may show lower levels of gene amplification.[38] Additionally, an increased amount of inhibitors from FFPE material cannot be excluded. Indeed, in the present study we observed that the archival DNA was amplified less efficiently than the fresh frozen sample DNA, even though all qMSP assays were designed to amplify short regions of approximately 100 bases. To compensate, at least in part, for this we increased the censoring threshold for the formalin-fixed paraffin embedded samples. Independent of this, the limited number of gastric ($n = 25$) as well as pancreatic ($n = 20$) samples included in the present study represent a limitation. However, high throughput array-based DNA methylation data from TCGA (http://cancergenome.nih.gov/) validated that all genes analyzed here display significantly higher methylation levels in tumor samples compared to matching controls (stomach: $n = 25$, pancreas: $n = 9$, Supporting Information Table S6).

In the present study, *CDO1* and *SFRP1* were the best performing individual methylation markers. Colorectal cancer specific methylation of *SFRP1* was identified already in 2002,[39] and has later also been demonstrated in other gastrointestinal malignancies, including stomach, liver, pancreas and cholangiocarcinoma.[40,41] The findings presented here are thus in line with previous reports. Interestingly, *CDO1* was only recently demonstrated to be methylated in colorectal cancer.[42] From analyses of one hundred cell lines derived from 17 different cancer types, we found *CDO1* to be frequently methylated not only across the gastrointestinal cancer cell lines but also in cell lines from ovary, uterus, lung, urinary bladder, lymphoma and prostate (data not shown). These findings are supported by a recent study by Brait *et al.*[35] who reported *CDO1* to be silenced in multiple human cancers. By combining microarray analysis and epigenetic treatment of cancer cell lines with several validation steps, *CDO1* was identified as methylated and downregulated in breast, bladder, colorectal, esophagus, lung and stomach cancer.[35] The methylation frequencies reported for colon and stomach cancer (91% and 87%, respectively), are highly similar to what we report here. We further detected elevated promoter methylation of *CDO1* in a significant proportion of the normal mucosa of cancer patients compared to cancer-free individuals. Interestingly, silencing of *CDO1* by DNA methylation was recently identified through a genome wide approach to have a driver function in tumorigenesis, and functional studies implied that methylation of this region is necessary for cell survival.[43] In line with this, the observed *CDO1* methylation in the normal mucosa samples from the cancer patients may be due to an epigenetic field defect, occurring before morphological changes in the colorectal mucosa. This indicates that *CDO1* is silenced early in the tumorigenesis which, in combination with its high sensitivity and specificity, suggests that it may be a suitable marker for early detection of colorectal cancer.

Doublecortin-like kinase 1 (*Dclk1*) expression was recently suggested to be an intestinal cancer stem cell specific biomarker based on functional studies in mouse models.[44] In contrast to this, we recently reported frequent (82%)

promoter methylation of one of the *DCLK1* reference sequences in colorectal cancers.[27] In the present study, *DCLK1* promoter methylation has been analyzed in additional cancer types. With a high methylation frequency also in gastric (96%) and pancreatic (50%) cancer, *DCLK1* may serve as a biomarker for gastrointestinal tumorigenesis.

This is, to the best of our knowledge, the first time *ZNF331* and *ZSCAN18* are reported to be hypermethylated in colorectal cancer. Yu *et al.* recently reported that zink finger protein 331 (*ZNF331)* is a candidate tumor suppressor gene mainly involved in gastric carcinogenesis.[45] Our data support this, in that 80% of the tumor samples analyzed in this study were found to be methylated. Zink finger and SCAN domain containing 18 (*ZSCAN18*) hypermethylation was recently reported in 32% of primary renal cell carcinomas, and RNAi knockdown of this gene was associated with an anchorage-independent growth advantage.[46]

Promoter DNA methylation is commonly associated with reduced or lost gene expression, and aberrant promoter methylation may be one mechanism used by cancer cells to silence specific genes, thereby providing them with a growth advantage. In this study, we observed a strong correlation between the presence of promoter methylation and reduced expression of all the analyzed genes across multiple cell lines from various cancer types. This negative correlation was further confirmed in colorectal cancer tissue samples for *ZNF331* and *ZSCAN18*, but not for *CDO1*, indicating that promoter methylation do not reduce expression of *CDO1*. However, a notably increased expression was observed for all genes in colon cancer cell lines after epigenetic drug treatment, suggesting that the lack of correlation for *CDO1* may be explained by the limited sample size of the unmethylated group. Moreover, in the rectum samples, where a higher percentage of the tumor samples were unmethylated, a significant negative correlation between expression and methylation was observed. Also for *ZNF331* and *ZSCAN18*, increased expression was seen after epigenetic treatment in colon cancer cell lines, confirming that the reduced expression of these genes most likely is caused by aberrant promoter methylation. No significant difference in expression of *CDO1* was found between colorectal cancers and samples taken from the normal mucosa of cancer patients. This is not surprising considering the high methylation frequency detected in the latter group. Even though the DNA promoter methylation of the genes analyzed in the present study seems to be functional in the sense that the gene expression is reduced, additional studies are warranted in order to conclude whether they are drivers or passengers in cancer development.

None of the biomarkers identified here amplify material from healthy controls, which means that they are highly cancer-specific with great potential as biomarkers. However, since they are methylated across several gastrointestinal cancer types, they are not cancer-type specific. The efficiency of a cancer test would obviously depend on the sample material used for analysis and whether DNA from the various cancer types is present in such sample material or not. Blood samples are systemic, and therefore have the potential of carrying biomarkers from the majority of cancer tissues. However, since the methylation levels (PMR values) in the present study generally are lower for pancreatic cancer and cholangiocarcinoma, these epigenetic aberrations may not efficiently be detected by a blood-based test. Site-specific sampling of material could additionally increase the cancer-type specificity. Fecal samples could in theory contain DNA from cancer cells in the stomach, pancreas and/or bile ducts. Contribution of colorectal cancer cells will, however, most likely be higher than contribution from other organs, increasing the likelihood of generating a more cancer-type specific or rather colorectal cancer specific test. In contrast, endoscopic retrograde cholangiography (ERC) derived biliary bile duct brush samples would be more pertinent for cholangiocarcinoma detection.

In conclusion, we report five genes that are highly methylated across gastrointestinal cancers. We additionally show that promoter DNA methylation of the analyzed genes is negatively correlated with gene expression in cancer cell lines, and that treatment with epigenetic drugs caused increased expression of the silenced genes. The frequent and specific methylation of these genes in colorectal cancer makes them promising biomarkers for detection of this malignancy.

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