

Novel target genes and a valid biomarker panel identified for cholangiocarcinoma

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Abbreviations: AUC, area under the ROC curve; CCA, cholangiocarcinoma; MSP, methylation-specific polymerase chain reaction; PMR, percent methylated reference; ROC, receiver operating characteristics; qMSP, quantitative methylation-specific polymerase chain reaction

Cholangiocarcinoma is notoriously difficult to diagnose, and the mortality rate is high due to late clinical presentation. CpG island promoter methylation is frequently seen in cancer development. In the present study, we aimed at identifying novel epigenetic biomarkers with the potential to improve the diagnostic accuracy of cholangiocarcinoma. Microarray data analyses of cholangiocarcinoma cell lines treated with epigenetic drugs and their untreated counterparts were compared with previously published gene expression profiles of primary tumors and with non-malignant controls. Genes responding to the epigenetic treatment that were simultaneously downregulated in primary cholangiocarcinoma compared with controls (n = 43) were investigated for their promoter methylation status in cancer cell lines from the gastrointestinal tract. Genes commonly methylated in cholangiocarcinoma cell lines were subjected to quantitative methylation-specific polymerase chain reaction in a total of 93 clinical samples (cholangiocarcinomas and non-malignant controls). *CDO1*, *DCLK1*, *SFRP1* and *ZSCAN18*, displayed high methylation frequencies in primary tumors and were unmethylated in controls. At least one of these four biomarkers was positive in 87% of the tumor samples, with a specificity of 100%. In conclusion, the novel methylation-based biomarker panel showed high sensitivity and specificity for cholangiocarcinoma. The potential of these markers in early diagnosis of this cancer type should be further explored.

Introduction

Cholangiocarcinoma (CCA) is the second most prevalent primary hepatobiliary malignancy and represents about 3% of all gastrointestinal cancers.^{1,2} According to the localization, CCAs are classified as either extrahepatic or intrahepatic. Both subtypes arise from the biliary epithelium, and they are difficult to diagnose. CCA is associated with inflammatory conditions of the biliary system, and patients with risk factors such as primary sclerosing cholangitis and liver fluke infestations have a higher risk of developing this malignancy.^{3–5} The generally late clinical presentation of CCA results in a high mortality, with a reported 5-y survival of only 5–15%.^{1,2}

The diagnosis of CCA remains challenging. The current clinical strategy for early diagnosis of malignancy includes a combination of various imaging modalities, as well as biliary brush

cytology and analysis of a few serum markers.^{3,6,7} However, CCA can often not be confirmed until a laparoscopy has been performed.³ The most commonly used molecular marker for detecting CCA is carbohydrate antigen 19–9.⁸ Unfortunately, this marker harbors limitations such as dependence of Lewis genotype and the possibility of elevated levels due to the presence of other gastrointestinal malignancies.⁹ Benign conditions such as acute cholangitis and cirrhosis may also result in elevated carbohydrate antigen 19–9 levels.^{10,11}

Tumor-specific molecular alterations, including both genetic and epigenetic aberrations, have been shown to play important roles in cancer development.^{12–15} Impaired epigenetic regulation, including aberrant DNA methylation, is frequently reported in cancers.^{12,15,16,17} In humans, DNA methylation occurs at the 5-position of cytosines in a CpG context.¹⁸ The bulk of the genome is methylated at most of these CpG sites, whereas dense

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CpG clusters, so-called CpG islands, are usually devoid of methylation. Aberrant DNA methylation of CpG islands located in the promoter region of genes is associated with transcriptional silencing.^{19,20} Loss of expression of essential tumor suppressor genes may lead to tumor development. Since aberrant DNA methylation also has been shown to be an early event in tumorigenesis,^{16,21,22,23} such targets may represent attractive biomarkers for early detection. Several genes known to be methylated in cancer in general, including *RASSF1A* and *CDKN2A* (*p16*), have also been analyzed for promoter methylation in CCA (see Table S1). However, an epigenome approach for detection of CCA target genes has not previously been undertaken. It should be noted that only those genes that are frequently methylated in tumors and are unmethylated in normal tissue represent promising biomarkers. CCAs can only be cured by radical resection or in selected cases by liver transplantation. Most frequently, patients have too advanced disease at presentation to be candidates for such treatment. The identification of suitable epigenetic CCA biomarkers with high sensitivity and specificity may facilitate cancer diagnostics at an early stage, perhaps performed upon biliary brush specimens, and thus contribute to increase survival of this patient cohort which presently carries such a poor prognosis.

The aim of the present study was to use an epigenome-wide approach²⁴ to identify a novel and suitable DNA methylation-based biomarker panel that may improve the diagnosis of CCA.

Results

Identification of candidate genes for promoter DNA methylation in cholangiocarcinomas. The experimental approach used to identify candidate genes for DNA methylation analysis in CCAs are summarized in Figure 1. From gene expression microarray analysis we observed 654 genes that were upregulated 2-fold or more in a minimum of four out of the six CCA cell lines after epigenetic drug treatment (5-aza-2'-deoxycytidine and trichostatin A). Sixty of these genes were simultaneously found to be downregulated in CCAs compared with non-malignant controls in previously published data sets (Fig. 2; Table S2).^{25,26} A CpG island was found in the promoter region of 43 of the candidates (including four gene-variants from three of these candidates) and these were regarded as potential DNA methylation target genes.

DNA promoter methylation analyses of candidate genes in cancer cell lines. The promoter methylation status of the 43 candidate genes was investigated in 24 cancer cell lines using MSP, and grouped according to their methylation frequency in CCA cell lines (Fig. 3). Genes in group I (n = 12) were frequently methylated ($\geq 5/6$; *SFRP1*, *TCF4*, *NAPIL2*, *DCLK1*, *CDO1*, *ZSCAN18* (NM_023926), *ZNF331* (NM_018555),

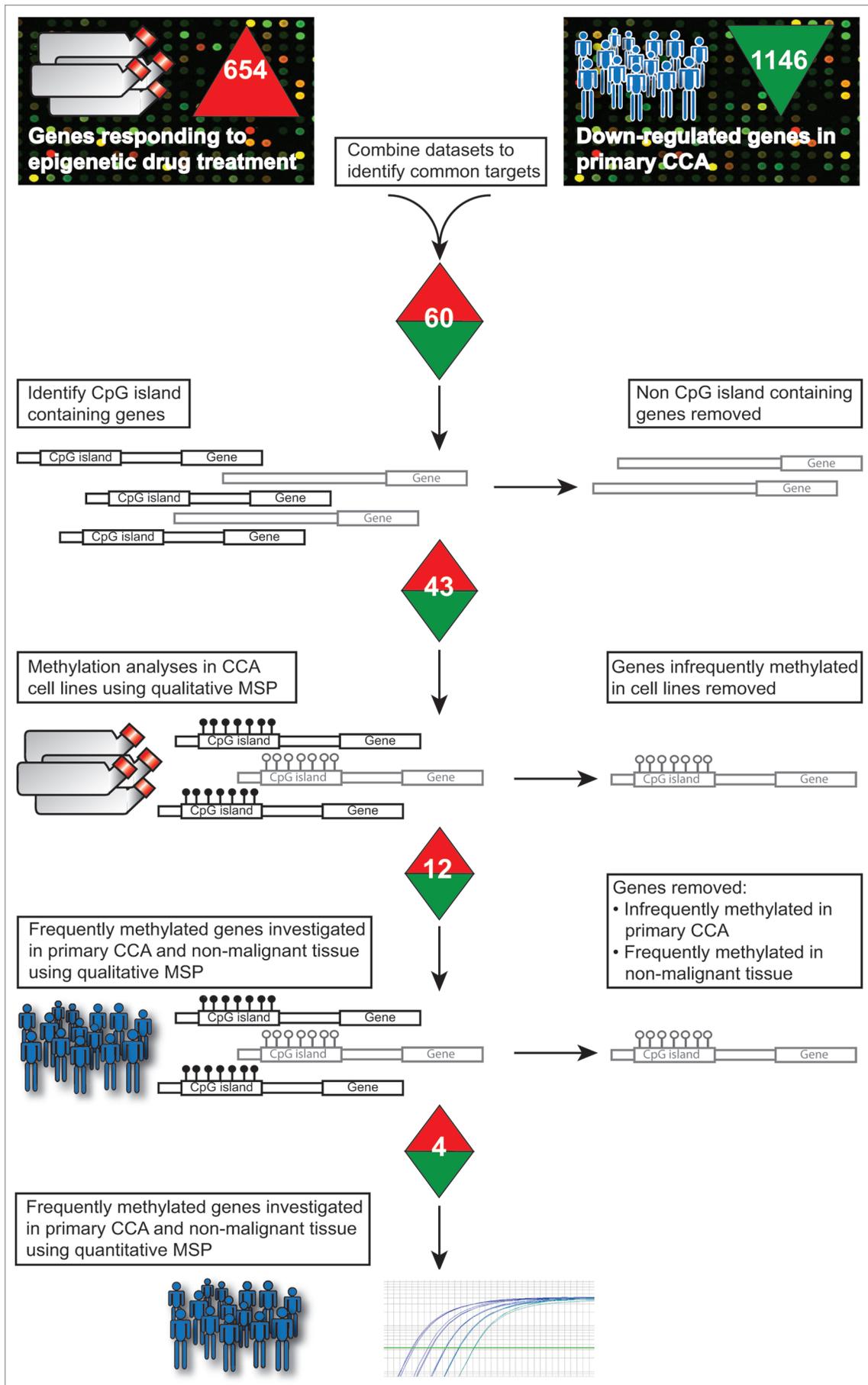
GREM1, *BEX4*, *TPM2*, *LHX6* and *FAM3B*. Genes in group II (n = 10) displayed intermediate methylation frequencies (1/6–4/6; *GNG11*, *ASRGL1*, *CRISPLD2*, *FKBP1B*, *PDE2A*, *REEPI*, *CSRPI*, *SLC46A3*, *INPP5A* and *MT1F*). The remaining 18 genes (group III) were unmethylated in all CCA cell lines [*ATF3*, *CALCOCO1*, *CLU*, *CTGF*, *DUSP5*, *EGR2* (NM_001136177), *FHL1*, *GPR124*, *HABP4*, *ID3*, *ITPR1*, *LMCD1*, *MLLT11*, *MTIX*, *MT2A*, *NR4A3*, *RNase4* and *SYT11*]. In subsequent analyses, the genes/transcript variants identified here will be referred to by their gene symbols. Genes/transcript variants *CXCL14*, *DPYSL3*, *EGR2* (NM_001136178), *STXBPI*, *ZNF331* (NM_001079906), *ZNF331* (NM_001079907) and *ZSCAN18* (NR_027135), were excluded from analysis based on the presence of a weak band in one of the following control reactions; the methylated reaction using normal blood, the unmethylated reaction using completely methylated DNA, or the methylated reaction using non-bisulfite treated DNA.

Interestingly, the methylation frequencies within groups I, II and III seemed comparable among the gastrointestinal cancer cell lines included in the present study, with the exception of *NAPIL2* and *LHX6*, which displayed little or no methylation in cell lines from hepatocellular carcinoma, and *ZNF331* and *TPM2*, which showed no to little methylation in cell lines from pancreatic cancer.

Qualitative DNA promoter methylation analysis of target genes in tissue samples. All group I genes were subjected to MSP analysis in 13 fresh-frozen CCA and 21 non-malignant controls. We observed methylation of *SFRP1*, *DCLK1*, *BEX4*, *NAPIL2*, *CDO1*, *ZSCAN18*, *GREM1*, *TCF4*, *ZNF331*, *LHX6*, *TPM2*, *FAM3B* in 85%, 75%, 69%, 69%, 62%, 31%, 23%, 23%, 23%, 8%, 8% and 0% in tumors, 19%, 38%, 33%, 33%, 0%, 0%, 6%, 0%, 0%, 0%, 43% and 0% in non-malignant controls (see Fig. S1). Note that for some genes low intensity methylated bands were detected among some of the non-malignant controls, which have been scored as weakly methylated. Although a few control samples were also scored as methylated, these band intensities were weaker than the bands seen among tumor samples. Thus, a quantitative methylation assay was assumed to discriminate more accurately between CCAs and non-malignant controls. Subsequently, gene promoters exhibiting more than 30% methylation in tumors (*SFRP1*, *DCLK1*, *CDO1* and *ZSCAN18*) were subjected to qMSP. *BEX4* and *NAPIL2* were excluded from further analysis since they displayed methylation in normal blood controls from females.

Quantitative DNA methylation analyses. Validation of promoter methylation status by direct bisulfite sequencing. To verify the promoter methylation status as assessed by MSP, the promoter region of *CDO1*, *DCLK1* and *ZSCAN18* were subjected to direct bisulfite sequencing in representative cancer cell lines. A good

Figure 1 (See opposite page). Epigenome-wide experimental approach for identifying methylated genes in cholangiocarcinomas. Six CCA cell lines were cultured with and without a combination of epigenetic drugs (5-aza-2'-deoxycytidine and trichostatin A). Array-elements responding to epigenetic drug treatment were compared with previously published downregulated genes in CCAs relative to cancer free tissue. Common genes, harboring a CpG island in the promoter region, were investigated for methylation in cancer cell lines from colon, bile duct, liver, gall bladder, and pancreas. Genes frequently methylated in CCA cell lines were subsequently investigated in patient material using MSP. The most promising candidates from this analysis were further evaluated by qMSP. Numbers indicate the number of genes fulfilling the selection criteria in each experimental step and which are subsequently subjected to further analyses.



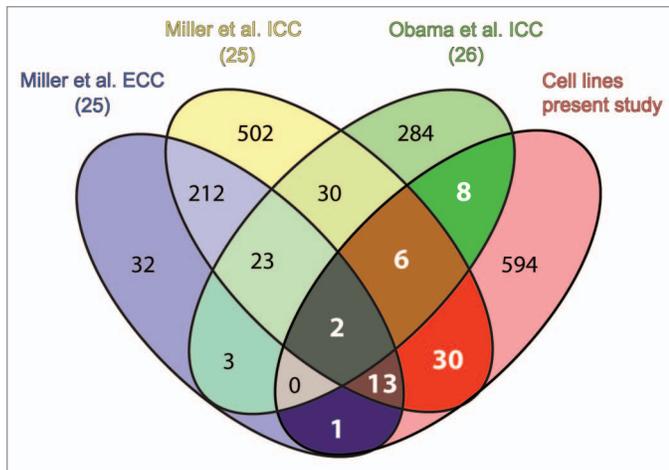


Figure 2. Venn diagram illustrating overlapping deregulated genes between cancer cell lines and cholangiocarcinomas. Using microarray analyses, a total of 654 genes were upregulated in CCA cell lines after epigenetic drug treatment (red circle, “Cell lines present study”). Sixty (white numbers in bold; Table S2) of these were simultaneously downregulated in at least one previously published data set of tumor compared with non-malignant controls (Miller et al.²⁵ and Obama et al.²⁶). ICC, intrahepatic cholangiocarcinoma; ECC, extrahepatic cholangiocarcinoma.

concordance was seen between the MSP and bisulfite sequencing results (see Fig. S2). The results were used to guide the design of the quantitative DNA methylation assays. *SFRP1* has previously been analyzed by qMSP^{27,28} and was therefore not included in the bisulfite sequencing analysis.

DNA methylation in fresh frozen and formalin-fixed tissues. From MSP analyses, genes methylated in 30% or more tumor samples (*CDO1*, *DCLK1*, *SFRP1* and *ZSCAN18*) were further investigated by qMSP in two sample series; fresh-frozen material comprising 13 CCAs and 21 non-malignant controls; and archival material (formalin-fixed and paraffin-embedded) comprising 26 tumor and 33 non-malignant controls. For the fresh-frozen sample series, we detected promoter methylation in 85%, 77%, 69% and 46% for *CDO1*, *ZSCAN18*, *SFRP1* and *DCLK1*, respectively, in tumors and no methylation in the non-malignant controls. By combining all four genes and scoring samples with methylation in minimum one out of the four genes as positive, 100% of the tumors and 0% of the non-malignant samples were methylation positive. The individual and combined performance of these genes was investigated by receiver operating characteristics curves (see Fig. S3). Combining the four genes (summarizing the PMR values) resulted in an area under the curve of 0.996 (95% asymptotic CI, N/A; asymptotic sig., 1.57E-6). Frequencies of methylation in the archival series were in general lower than the findings in the fresh frozen sample set, although not statistically significant. The four genes *CDO1*, *SFRP1*, *ZSCAN18* and *DCLK1* displayed promoter methylation frequencies of 73%, 54%, 42% and 42%, respectively, in tumors, whereas no methylation was observed in the non-malignant controls. The combined panel in archival tissue was methylation positive in 81% of the tumors. The resulting area under the curve for this sample

set was 0.904 (asymptotic 95% CI; 0.811–0.997, asymptotic sig., 1.17E-7) (see Fig. S3).

For the total series of tumors (n = 39), *CDO1*, *SFRP1*, *ZSCAN18* and *DCLK1* displayed promoter methylation frequencies of 77%, 59%, 54% and 44%, respectively. The biomarker panel reached a sensitivity of 87% and specificity of 100%, yielding an area under the curve of 0.924 asymptotic 95% CI, 0.854–0.994; asymptotic sig., 3.79E-12; Fig. 4).

Discussion

In the present study, we have identified *CDO1*, *DCLK1* and *ZSCAN18* as novel frequently methylated genes in cholangiocarcinoma, and confirmed frequent methylation of the *SFRP1* gene.^{27,28} Tissue samples from carcinoma-free individuals were unmethylated for the same genes, indicating that the promoter methylation was tumor specific. The high sensitivity and specificity of *CDO1*, *DCLK1* and *ZSCAN18* underscore their suitability as biomarkers for cholangiocarcinoma. Including *SFRP1*, the resulting biomarker panel achieved a sensitivity and specificity of 100% in fresh frozen material.

In lack of a validation series (due to the low incidence of cholangiocarcinomas), we included a second sample series consisting of archival formalin-fixed and paraffin-embedded samples. Although the sensitivity decreased somewhat in the archival material, the combined sensitivity (87%) and specificity (100%) for both sample series indicated that the present biomarker panel has the potential to improve the diagnostic accuracy of CCA compared with existing clinical approaches.^{3,6,7} It should however be noted that the number of samples analyzed represents a limitation to the present study. Additional samples would increase the statistical power, and validation of the presented biomarker panel is therefore warranted.

In order to detect cholangiocarcinomas irrespective of subclassification and disease background, the tumor material used in this study included both extrahepatic and intrahepatic lesions with and without concomitant primary sclerosing cholangitis (PSC). A pool of non-malignant liver diseases (including PSC) as well as disease free biopsies served as controls. The resulting high sensitivity and specificity underscore that the biomarker panel can discriminate well between cancer and various non-cancerous lesions.

Epigenome-wide expression profiling has also previously been used to identify potential epigenetic markers in several tumor types, including pancreas and prostate.^{29,30} Using a similar approach, we recently identified promising epigenetic markers for early detection of bladder³¹ and colorectal cancer.^{24,32,33} Current advances in technology, including DNA methylation arrays and high throughput sequencing, have expanded the range of methods that can be used for such biomarker identification. However, based on the diagnostic potential of the already identified biomarkers in bladder and colorectal cancer, we chose to use the same approach also in the present study.

From previous cancer cell line analyses we have observed that biomarkers identified in colorectal cancer were present also in other cancers of the gastrointestinal tract, although at different

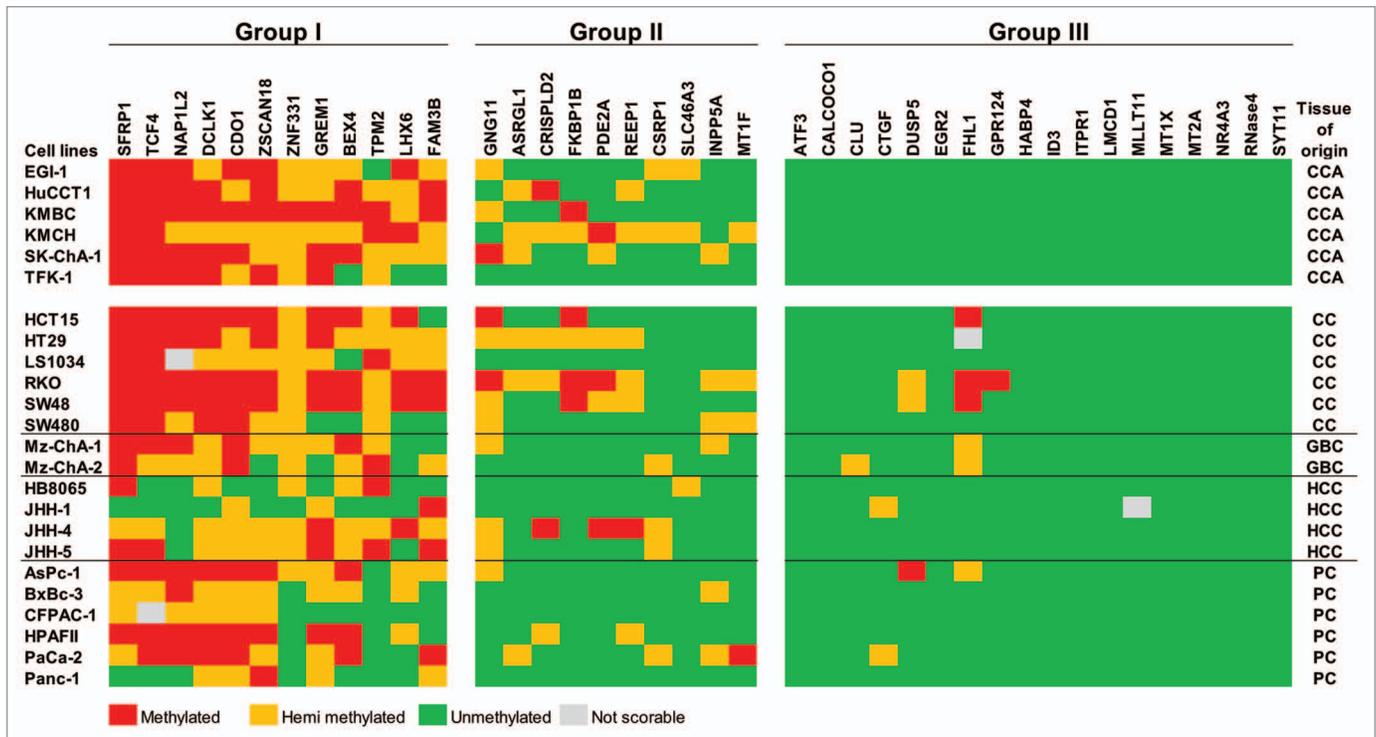


Figure 3. Summary of promoter methylation status in cancer cell lines. Forty-three genes were analyzed by MSP. Three genes were excluded after control reactions. The remaining 40 genes were grouped according to their methylation frequency in CCA cell lines. Group I, frequently methylated (minimum five out of six cell lines); group II, intermediately methylated (from one to four cell lines); group III, unmethylated. Accession numbers corresponding to gene symbols are listed in Table S5. CCA, cholangiocarcinoma; CC, colon cancer; GBC, gall bladder carcinoma; HCC, hepatocellular carcinoma; PC, pancreatic cancer.

frequencies.³³ Subsequently, in the present study, cell lines from several hepatopancreatobiliary tumors as well as colon cancer were included (Fig. 3). As expected, we observed similarities in methylation frequencies across the majority of cancer cell lines, indicating that the genes may be aberrantly methylated also in tissue samples from other gastrointestinal tumors. This could potentially represent a cancer-specificity problem in a future test. However, by using minimally invasive material sampled in the bile ducts (such as bile or biliary brush cytology specimens), one can restrict the source of cancer cells to the tissue of interest. Such a clinical proof-of-principle was recently published, demonstrating that epigenetic biomarkers with high sensitivity and specificity could be detected in the bile of cholangiocarcinoma patients.³⁴

One of the four biomarkers presented here, *SFRP1*, has previously been investigated as a potential epigenetic biomarker in several cancers, including cholangiocarcinoma.^{27,28,35,36} Secreted frizzled-related protein (SFRP) family members act as modulators of the Wnt-pathway, and methylation of these promoter regions can lead to deregulation of this pathway and subsequent cancer development.³⁷ The methylation frequencies (64%) published for *SFRP1* by Uhm et al.²⁸ and Sriraksa et al.²⁷ are in the same range as presented here.

Cysteine dioxygenase, type 1 (*CDO1*) is reported to be highly expressed in the liver.³⁸ It is involved in initiation of metabolic pathways related to pyruvate and sulfurate compounds, including taurine, which is a major constituent of bile. Methylation of

the *CDO1* promoter was recently shown to be a strong marker for distant metastasis in lymph node positive, estrogen receptor positive breast cancer patients.³⁹ In addition, *CDO1* has been indicated to be epigenetically deregulated in colorectal cancer, lung cancer and Wilms tumor.⁴⁰⁻⁴² Our results supported these findings and showed for the first time that the promoter methylation of *CDO1* also may play a role in cholangiocarcinoma.

Doublecortin like kinase 1 (*DCLK1*) is a microtubule-associated kinase that can undergo autophosphorylation. To our knowledge, this is the first study reporting promoter methylation of this gene in cancer. However, previous studies have proposed the *DCLK1* protein expression as a marker for intestinal stem cells with a role in the epithelial-mesenchymal transition.^{43,44} The identified promoter methylation suggested a silencing of this gene, and further studies should be performed to clarify the role of aberrantly regulated *DCLK1* in cholangiocarcinoma.

Morris and colleagues recently reported on a putative tumor suppressor function of zinc finger and SCAN domain-containing protein 18 (*ZSCAN18*) in renal cell carcinoma.⁴⁵ In the present study we showed that *ZSCAN18* is slightly more frequently methylated in cholangiocarcinomas compared with renal cancer (32%).

Brush cytology samples can be obtained from biliary strictures during endoscopic retrograde cholangiography. The utility of routine cytological assessment of such samples is however hampered by limited sensitivity and specificity. The addition of molecular

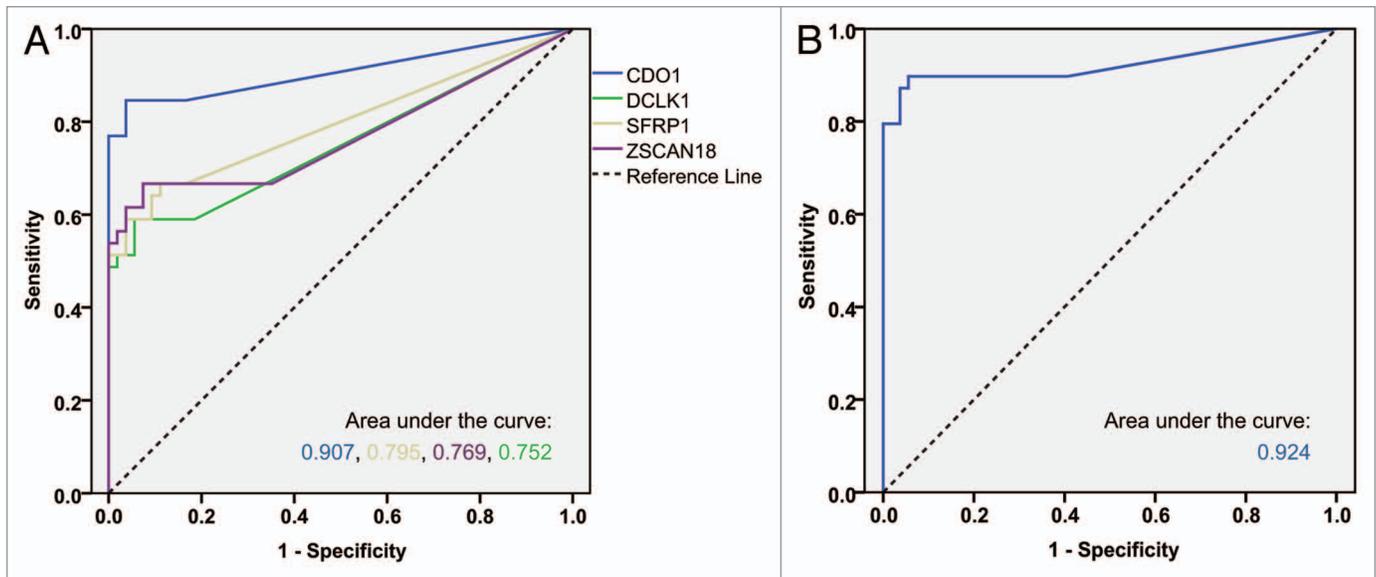


Figure 4. Receiver operating characteristics curves for individual and combined genes in cholangiocarcinomas and non-malignant samples. The panels depict the resulting area under the ROC curve based on the PMR values for (A) individual biomarkers and (B) the biomarker panel.

techniques like fluorescence in situ hybridization (FISH) may improve the diagnostic performance of brush cytology to some extent,⁴⁶ but still more accurate tests are needed to enable an early and reliable diagnosis of cholangiocarcinoma. Considering the high sensitivity and specificity DNA methylation cancer biomarkers have achieved across several cancer types so far, including early stage cancers,^{33,47-49} we believe that such markers in the near future could be of value also in the detection of cholangiocarcinomas.

In conclusion, we have identified four commonly methylated genes (*CDO1*, *DCLK1*, *SFRP1* and *ZSCAN18*) in CCA, of which three (*CDO1*, *DCLK1* and *ZSCAN18*) have not previously been described in this cancer type. The combined performance of this biomarker panel reached 87% sensitivity and 100% specificity across fresh frozen and archival material. Further studies should be performed in minimally invasive samples, e.g., bile, biliary brush cytology specimens and/or blood in order to evaluate if the presented panel can contribute in minimally invasive CCA diagnostics.

Methods

Experimental approach. The step-wise experimental approach used in the present study is illustrated in Figure 1. Briefly, genes responding to epigenetic drug treatment in cancer cell lines were compared with a list of genes downregulated in CCA samples vs. non-malignant tissue. Responding and simultaneously downregulated genes harboring a CpG island in the promoter region were subjected to qualitative methylation analysis in cancer cell lines. The most frequently methylated genes were further subjected to qualitative and subsequently quantitative methylation analyses in patient material.

Cancer cell lines. Twenty-four cancer cell lines were analyzed, including bile duct (n = 6; EGI-1, HuCCCT1, KMBC, KMCH-1, SK-ChA-1 and TFK-1), colon (n = 6; HCT15, HT29, LS1034,

RKO, SW48 and SW480), pancreas (n = 6; AsPc-1, BxBc-3, CFPAC-1, HPAFII, PaCa-2 and Panc-1), liver (n = 4; HB8065, JHH-1, JHH-4 and JHH-5) and gallbladder (n = 2; Mz-ChA-1 and Mz-ChA-2) cancers. The cell lines were cultured according to the manufacturer's guidelines and the conditions are summarized in Table S3. All cell lines were harvested before reaching confluence.

Cell line authentication was performed using the AmpFLSTR Identifier PCR Amplification Kit (Applied Biosystems) according to the manufacturer's protocol. Samples were run on an AB Prism 3730 and analyzed in GeneMapper (Applied Biosystems). For commercially available cancer cell lines the genotypes were compared with previously published data (ATCC). Results for non-commercial cell lines are listed in Table S4.

The six cholangiocarcinoma cell lines were subjected to treatment with a combination of the demethylating drug 5-aza-2'-deoxycytidine (1 mM for 72 h; Sigma-Aldrich, A3656) and the histone deacetylase inhibitor trichostatin A (1 mM added the last 12 h; Sigma-Aldrich, T1952). Untreated controls were cultured in parallel.

Patient samples. Fresh frozen material. Thirteen bile duct carcinomas were derived from patients undergoing surgery at Oslo University Hospital Rikshospitalet and Imperial College. Samples were snap-frozen immediately after surgery and stored at -80°C. Carcinomas were embedded in Tissue-Tek (Sakura Finetek) according to the manufacturer's protocol, and subsequently subjected to cryo-sectioning and hematoxylin and eosin staining before they were evaluated by an expert pathologist. All carcinoma samples displayed > 5% tumor cells. Twenty-one samples from non-malignant liver diseases, including autoimmune hepatitis (n = 2), alcohol related liver disease (n = 5), cryptogenic cirrhosis (n = 1), hemochromatosis (n = 1), primary biliary cirrhosis (n = 3) and primary sclerosing cholangitis (n = 9) were used as non-malignant controls. The non-malignant biopsies were

Table 1. Assays used for quantitative methylation-specific polymerase chain reaction (qMSP)

Assay	Sense primer	Antisense primer	Probe	Frg. size (bp)
ALU qMSP*	GGT TAG GTA TAG TGG TTT ATA TTT GTA ATT TTA GTA	ATT AAC TAA ACT AAT CTT AAA CTC CTA ACC TCA	6FAM-CCTACCTTAACCTCCC- MGB	98
CDO1 qMSP	CGA ATT ATA GCG GCG GAG GT	AAA TCG CGT AAA CTC CGC G	6FAM-CGTTAGGTCGGGCGGT- MGB	101
DCLK1 qMSP	GCG CGT ACG CGG AGG	CGA CGA CGA ACG CGC T	6FAM-CGGGAGGGCGGTGA- MGB	86
SFRP1 qMSP#	GAA TTC GTT CGC GAG GGA	AAA CGA ACC GCA CTC GTT ACC	6FAM-CGTACCCGACGCGAA- MGB	70
ZSCAN18 qMSP	CGC GGT ATA GTT TCG CGG TAT	CGC GAT AAC GAC CGA CAA A	6FAM-CGTAGTTCGCGGTGAGG- MGB	84

*qMSP assay was obtained from Weisenberger and colleagues.⁵⁰ #qMSP assay was modified from Rawson and colleagues.⁵¹

obtained from the peripheral liver region of patients undergoing liver transplantation at Oslo University Hospital, Rikshospitalet.

Archival material. The archival sample series comprised 26 CCA and 33 non-malignant formalin-fixed, paraffin-embedded samples obtained from the Department of Pathology, Oslo University Hospital. The tissue specimens were routinely stained with hematoxylin and erythrosine. All carcinoma samples included displayed > 5% tumor cells. Tissue from non-malignant liver disease patients, including autoimmune hepatitis (n = 4), alcohol related liver disease (n = 1), primary biliary cirrhosis (n = 4) and primary sclerosing cholangitis (n = 21), as well as from disease free patients (n = 3) were included as non-malignant controls.

The project has been approved by the regional committee for research ethics in southeastern Norway (S-08512b2008/16133) and all included patients have given informed consent.

Gene expression microarray analyses of cancer cell lines. RNA from six CCA cell lines and their epigenetic drug treated counterparts were subjected to gene expression microarray analyses (Applied Biosystems). See supplemental appendix A for protocol details. Genes upregulated at least 2-fold after 5-aza-2'-deoxycytidine and trichostatin A treatment in a minimum of four of the analyzed cancer cell lines were considered to be potential targets for promoter DNA methylation.

Microarray gene expression data sets from cholangiocarcinoma patients and healthy controls. In order to increase the likelihood of identifying putative methylated genes in CCA, the gene list generated from the microarray approach in CCA cell lines was compared with gene lists generated from published and available gene expression microarray data comparing CCAs with non-malignant controls.^{25,26} Only responding genes from the cell line approach that were simultaneously downregulated in CCA compared with non-malignant controls were considered methylation candidates and subjected to further analyses.

DNA promoter methylation analyses. Candidate genes were analyzed for the presence of a CpG island in the promoter region and appropriate DNA methylation assays were designed and optimized. Bisulfite treatment was performed prior to DNA methylation analyses. Qualitative methylation-specific polymerase chain reaction (MSP) was performed in all cancer cell lines. Genes

methylated in a minimum of five out of six CCA cell lines were further subjected to MSP in fresh frozen tissue samples (n = 34). The best performing genes from the qualitative analysis (*CDO1*, *DCLK1* and *ZSCAN18*) were subjected to direct promoter bisulfite sequencing. Primer sequences and location, amplicon length, MgCl₂ concentration and annealing temperatures for MSP and bisulfite sequencing are listed in Table S5. The methylation status of the three abovementioned genes, in addition to the previously reported *SFRP1*, was finally assessed in both fresh frozen and archival patient material using quantitative methylation-specific polymerase chain reaction (qMSP). Sequences are listed in Table 1. For a detailed description of the abovementioned technical procedures see Supplemental Materials.

Statistical analysis. For statistical analyses, PASW 18.0 (SPSS) was used. Pearson's chi-square and Fisher's exact tests were used for categorical variables. Student's t-test and Mann-Whitney U test were used to investigate potential relationships between tumor DNA methylation and patient age. To evaluate the suitability of the methylated target genes to separate CCA from non-malignant controls, receiver operating characteristics curves were generated using the individual percentage methylated reference (PMR) values. Similarly, the combined performance of the candidate genes as a panel was evaluated using the sum of the PMR values. P values < 0.05 were considered statistically significant.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Supplemental Materials

Supplemental materials may be found here:
www.landesbioscience.com/journals/epigenetics/article/22191

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