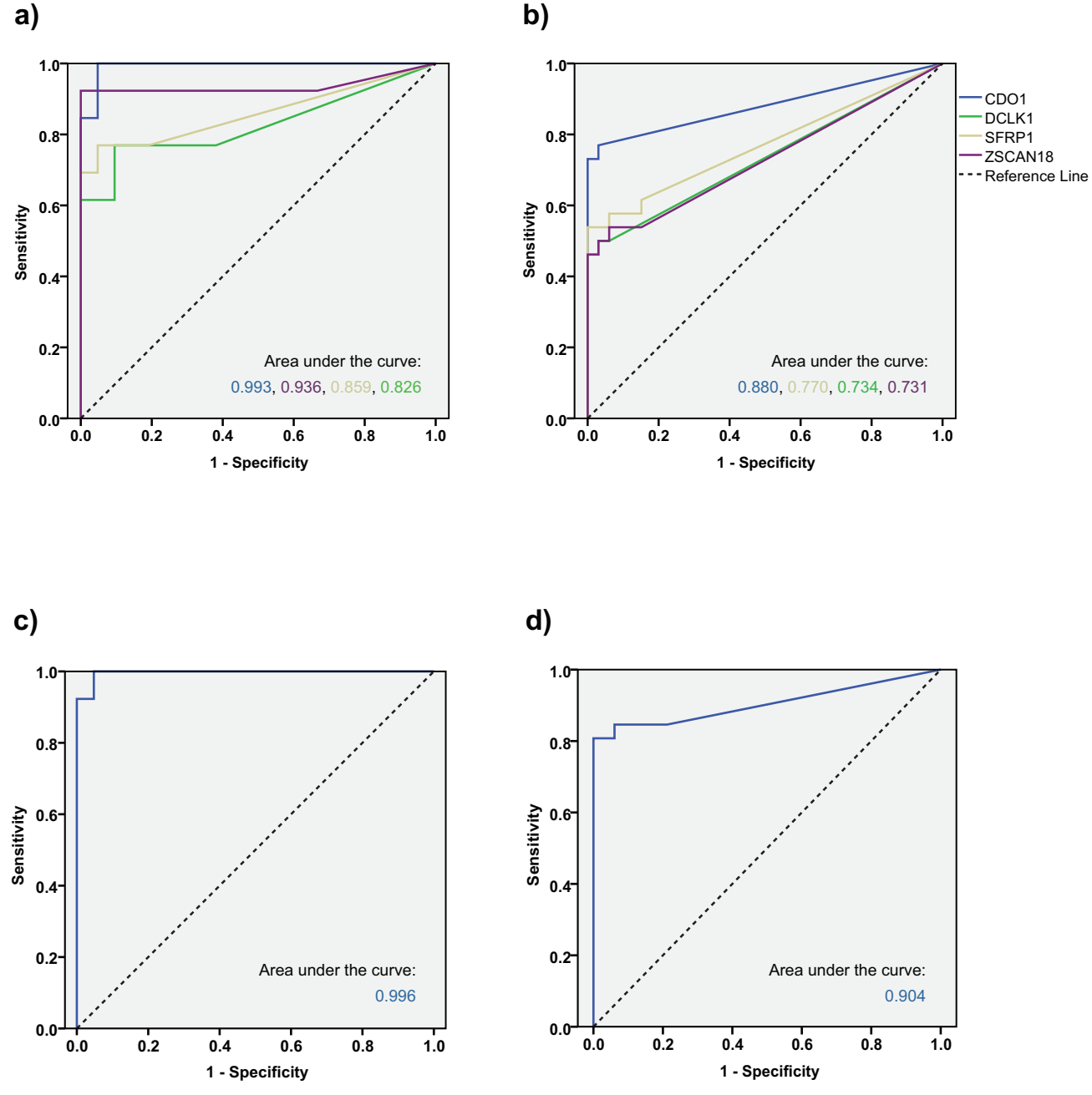
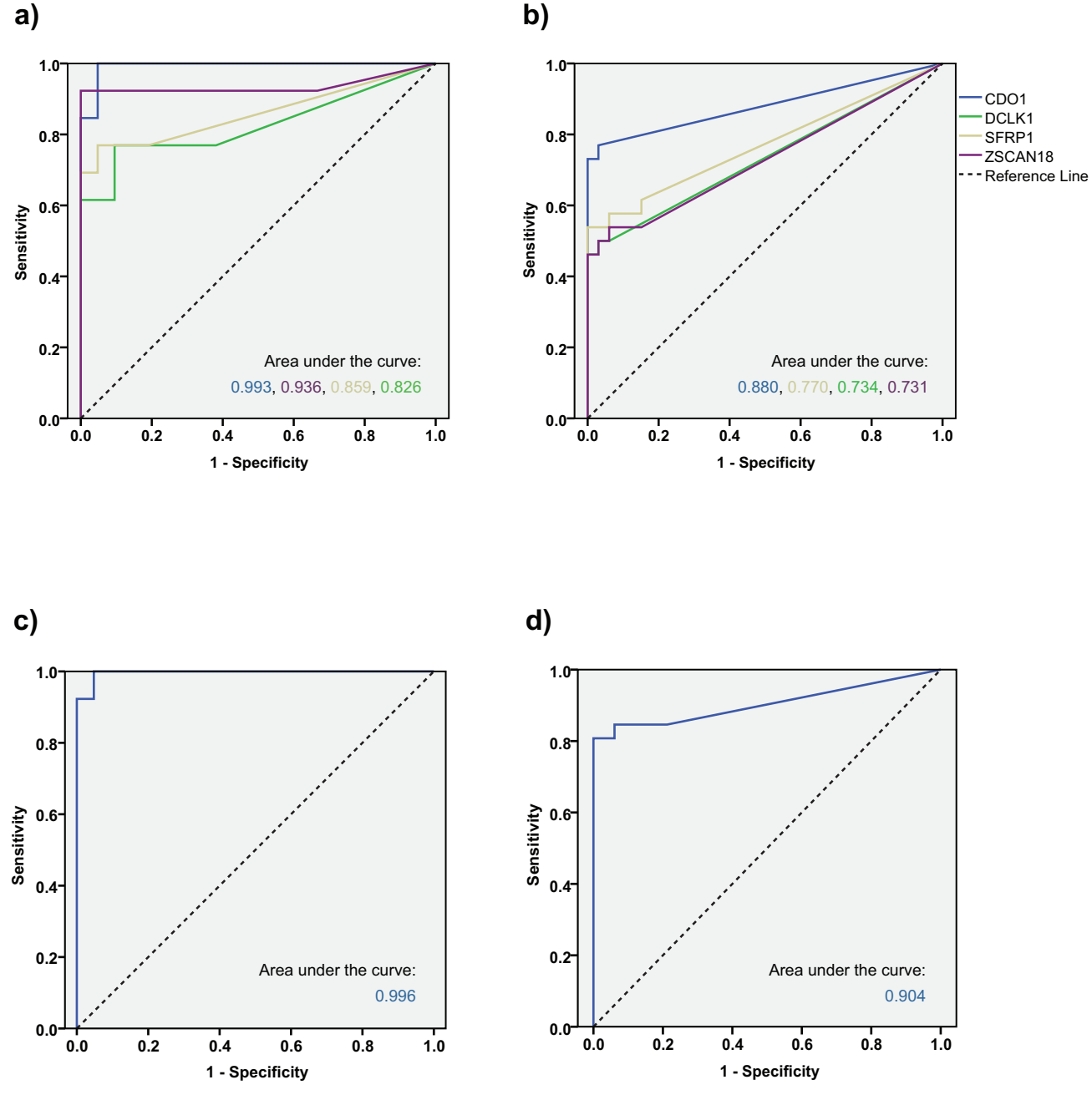
Supplemental Material to:

Andresen K, Boberg KM, Vedeld HM, Honne H, Hektoen M, Wadsworth CA, et al. Novel target genes and a valid biomarker panel identified for cholangiocarcinoma, Epigenetics 2012; 7(11); http://dx.doi.org/10.4161/epi.22191

http://www.landesbioscience.com/journals/epigenetics/article/22191


a)
b)
c)
d)
Sensitivity
1 - Specificity
0.0
0.2
0.4
0.6
0.8
1.0
Area under the curve:
0.993, 0.936, 0.859, 0.826
Area under the curve:
0.880, 0.770, 0.734, 0.731
CDO1
DCLK1
SFRP1
ZSCAN18
Reference Line
Area under the curve:
0.996
Area under the curve:
0.904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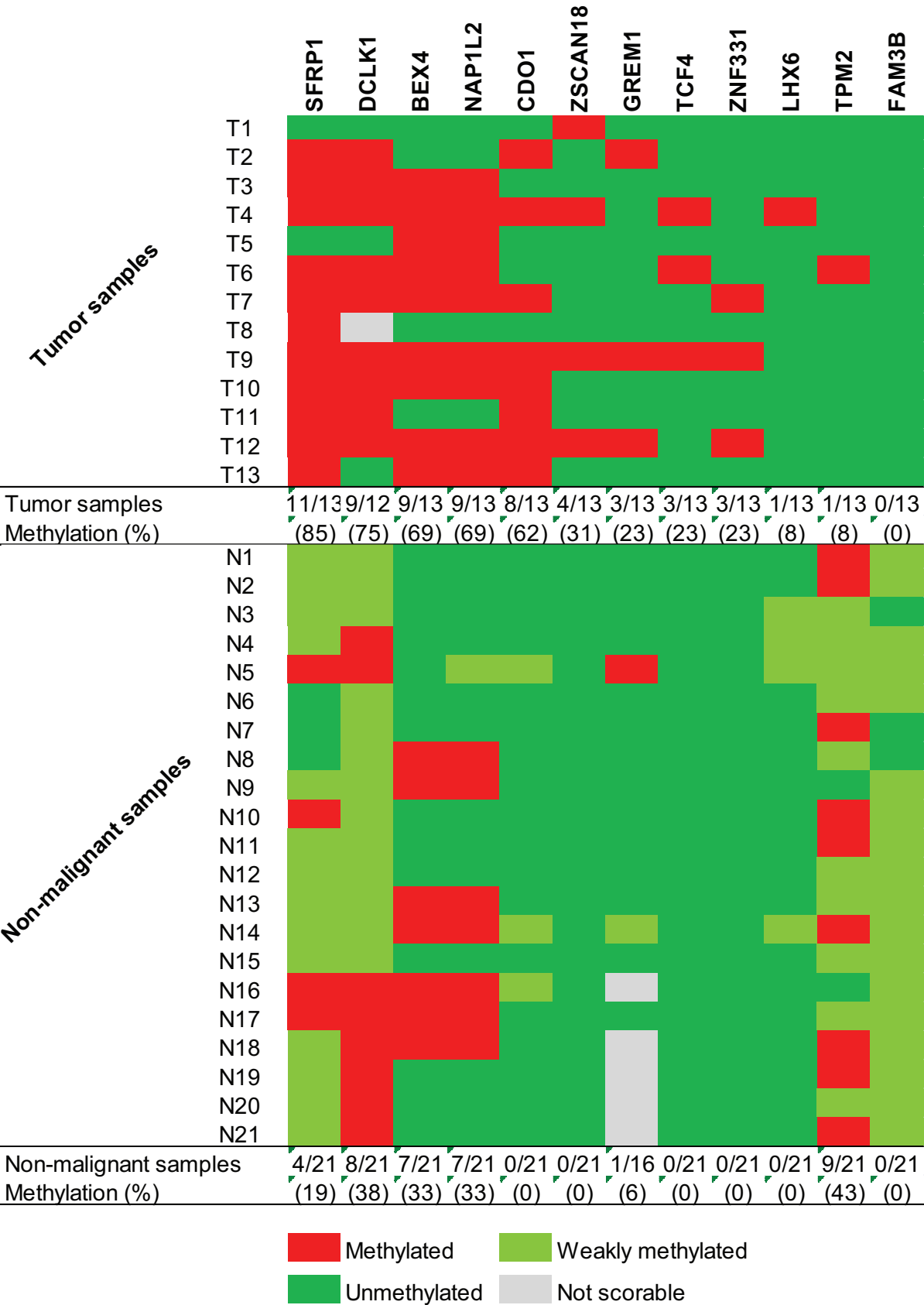
SFRP1
DCLK1
BEX4
NAP1L2
CDO1
ZSCAN18
GREM1
TCF4
ZNF331
LHX6
TPM2
FAM3B
Tumor samples
T1
T2
T3
T4
T5
T6
T7
T8
T9
T10
T11
T12
T13
Tumor samples Methylation (%)
11/13 (85)
9/12 (75)
9/13 (69)
9/13 (69)
8/13 (62)
4/13 (31)
3/13 (23)
3/13 (23)
3/13 (23)
1/13 (8)
1/13 (8)
0/13 (0)
Non-malignant samples
N1
N2
N3
N4
N5
N6
N7
N8
N9
N10
N11
N12
N13
N14
N15
N16
N17
N18
N19
N20
N21
Non-malignant samples Methylation (%)
4/21 (19)
8/21 (38)
7/21 (33)
7/21 (33)
0/21 (0)
0/21 (0)
1/16 (6)
0/21 (0)
0/21 (0)
0/21 (0)
9/21 (43)
0/21 (0)
Methylated
Weakly methylated
Unmethylated
Not scorable

Supplementary Appendix

Supplement A

Nucleic acid isolation

For cancer cell lines, DNA was isolated using a standard phenol/chloroform procedure and total RNA was isolated using Trizol (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. RNA quality was assessed using a 2100 bioanalyzer (Agilent Technologies, Palo Alto, CA). For fresh frozen samples, DNA was extracted from approximately 25 mg of tissue using the AllPrep DNA/RNA kit (Qiagen Inc, Valencia, CA). For archival material, DNA was isolated from five sections of paraffin embedded tissue à 20 µm, using the QIAamp DNA kit (Qiagen). Nucleic acid concentration was determined using the ND-1000 Nanodrop (NanoDrop Technologies, Wilmington, DE).

Description of the gene expression microarray analysis

Standard one-round amplification was performed using the NanoAmp RT-IVT Labeling Kit (Applied Biosystems) according to the manufacturers' protocol. Briefly, cDNA was synthesized from one µg total RNA in an oligo dT primed reaction. Labeled complementary RNA (cRNA) was obtained from double-stranded cDNA in the presence of digoxigenin (DIG)-UTP in an *in vitro* labeling reaction. Samples were hybridized to gene expression microarrays (Human Genome Survey Microarray V2.0, Applied

Biosystems) containing 32,878 oligonucleotide probes representing 29,098 individual genes. Chemoluminescense was measured using the AB1700 Chemoluminescense Analyzer (Applied Biosystems) after incubating the array with alkalic phosphatase-linked digoxygenin antibody. Probe signals were post-processed and quantile normalized using the R-script (R 2.5.0) "ABarray" file 1.2.0 and Bioconductor (www.bioconductor.org) and further analyzed in Excel (Microsoft office, version 2007). Array elements with a signal-to-noise ratio <3, and/or a flag value >8191 were discarded from further analysis.

Supplement B

CpG island search

Potential candidate genes for DNA methylation were analyzed for the presence of a CpG island in the promoter region using the CpG island searcher algorithm.[17] Default criteria were used for CpG island detection.[18]

Bisulfite treatment of DNA

Bisulfite treatment of DNA results in the conversion of unmethylated but not methylated cytosines to uracil.[19,20] DNA (1.3 µg) was bisulfite treated using the EpiTect bisulfite kit (Qiagen) according to manufacturers' protocol. Desulfonation and washing steps were performed using a QIAcube (Qiagen) and the bisulfite treated DNA was eluted in 40 µl elution buffer.

Qualitative methylation-specific polymerase chain reaction (MSP)

MSP primers were designed in close proximity of the transcription start site, according to the Human Genome browser (genome.ucsc.edu), using the Methyl Primer Express Software v1.0 (Applied Biosystems). Primers were purchased from MedProbe (MedProbe, Oslo, Norway). Two pairs of primers were used to amplify the loci of interest, one specific for methylated and one specific for unmethylated template. The MSP reaction consisted of one unit HotStarTaq DNA polymerase (Qiagen), 1×PCR buffer (Tris-Cl, $KCl(NH_4)_2SO_4$, 15 mM $MgCl_2$; pH 8.7; Qiagen), 200 µM of each dNTP (Amersham Biosciences, Piscataway, NJ, USA), primers (800 pM each) and 24 ng of bisulfite treated template DNA in a total volume of 25 µl. All MSP reactions were optimized with respect to $MgCl_2$ concentration, annealing temperature and elongation time (Supplementary Table 4). In addition, the following control experiments were performed for each MSP assay; bisulfite treated and non-bisulfite treated normal lymphocyte DNA was applied in the methylated reaction, and completely methylated DNA was applied in the unmethylated reaction. Thermal cycling (Tetrad 2, Bio-Rad, CA, USA) was performed at 95C° for 15 minutes and 35 subsequent cycles (30 seconds at 95°C, 30 seconds at variable annealing temperatures (Supplementary Table 4), and 30-60 seconds elongation at 72°C) with a final extension of seven minutes at 72°C. Human placental DNA (Sigma Chemical Co, St Louis, MO) treated *in vitro* with *SssI* methyltransferase (New England Biolabs Inc., Beverly, MA) was used as positive control for the methylated reaction and DNA from normal lymphocytes was used as a positive control for the unmethylated reaction. Water, replacing the template, was used as a negative control in both reactions. MSP products were mixed with five µl gel loading buffer (1 x TAE buffer, 20% Ficoll; Sigma Aldrich, and 0.1% xylen cyanol; Sigma Aldrich) and loaded onto a

2% agarose gel (BioRad, Hercules, CA, USA) in 1 x TAE buffer with ethidium bromide (Sigma Aldrich). Electrophoresis was performed at 200V for 25 minutes before MSP products were visualized by UV irradiation using a Gel Doc XR+ (BioRad). All results were verified by a second independent round of MSP and scored independently by two authors (KA and HMV). In cases of discrepancies, a third round of analysis was performed. Scoring was performed using the methylated band intensities of positive controls as a reference. In tissue samples, the methylated band intensities were scored on a scale from zero to five. Tumor samples were only considered methylated when band intensities were equal to or stronger than three. For non-malignant samples, methylated band intensities equal to or stronger than three were scored as methylated, while methylated band intensities equal to or below two were scored as weakly methylated. Only non-malignant samples with the absence of a band in the methylated reaction (intensity 0) were scored as unmethylated.

Direct DNA bisulfite sequencing

Fourteen cancer cell lines (colon, n=6; cholangiocarcinoma, n=6 and gallbladder carcinoma, n=2) were subjected to bisulfite sequencing. DNA bisulfite primers were designed using the Methyl Primer Express Software v1.0 (Applied Biosystems) to cover the area amplified by the MSP. The experimental procedure has been published previously.[21] Briefly, fragments were amplified for 35 cycles using HotStarTaq (Qiagen) and purified using ExoSAP-IT, according to the protocol of the manufacturer (GE Healthcare, USB Corporation, Ohio, USA). Sequencing was performed using the dGTP BigDye Terminator Cycle Sequencing Ready Reaction kit on the AB Prism 3730 (Applied Biosystems). Bisulfite-treated completely methylated DNA (CpGenome Universal Methylated DNA,

Millipore, MA, USA) and DNA from normal lymphocytes served as positive and negative controls, respectively. In accordance with a previous report[22], the amount of methylcytosine of each CpG site was calculated by the peak height ratio of the cytosine signal versus the sum of cytosine and thymine signal. Methylation of individual CpG sites was determined after the following criteria: 0-0.2 was unmethylated, 0.21-0.8 was partially methylated and 0.81-1.0 was scored as methylated.

Quantitative methylation-specific polymerase chain reaction (qMSP)

Primers and probes were designed using Primer Express v3.0 and purchased from Medprobe and Applied Biosystems, respectively. All qMSP reactions were carried out in triplicates in 384-well plates. The total reaction volume was 20 µl, and included 0.9 µM of each primer, 0.2 µM probes (labelled with 6-FAM and a non-fluorescent quencher), 30 ng bisulfite treated template and 1x TaqMan Universal PCR master mix NoAmpErase UNG (Applied Biosystems). Amplification was performed at 95°C for 15 minutes before 45 cycles of 15 seconds at 95°C and 1 minute elongation at 60 °C, using the TaqMan 7900HF (Applied Biosystems). Bisulfite-converted completely methylated DNA (Millipore) served as a positive control and was also used to generate a standard curve by 1:5 serial dilutions (32.5 – 0.052 ng). The ALU-C4 gene[23] was used for normalization. In addition, bisulfite treated and untreated DNA from normal lymphocytes, and water blanks were used as negative controls.

All samples were censored after cycle 35 (in accordance with Applied Biosystems protocol) and the median quantity value was used for further processing. Briefly, percent methylated reference (PMR) was calculated by dividing the GENE:ALU ratio in the sample by

the GENE:ALU ratio of the positive control (completely methylated DNA) and multiplying by 100. To ensure high specificity, individual fixed thresholds were established for each assay (one for fresh frozen material and one for archival material), using the integer above the highest PMR value across the normal samples. Samples with higher PMR values than the thresholds were scored as methylation positive. For *CDO1*, *DCLK1*, *SFRP1,* and *ZSCAN18* the thresholds for the fresh frozen sample series were 1, 2, 1, and 1, respectively. For the archival sample series, thresholds were set at 2, 2, 5, and 3, respectively.

Supplementary Figures

Supplementary Figure 1. Methylation frequencies in patient material assessed by qualitative methylation-specific polymerase chain reaction (MSP). The twelve group I genes (see figure 2) were investigated in a fresh frozen sample set. Methylation frequencies are indicated for tumor and non-malignant samples. The weakly methylated bands observed among some of the non-malignant samples (light green color) have not been included as methylated in the calculated methylation frequencies.

Supplementary Figure 2. Direct bisulfite sequencing of *CDO1*, *DCLK1* and *ZSCAN18* verified the methylation status as assessed by MSP and was concordant with qMSP. a) *CDO1*. b) *DCLK1*. c) *ZSCAN18*. For all panels, the upper line represents the individual CpG sites (vertical bars) in the fragment amplified by the bisulfite sequencing primers. Transcription start site is denoted by +1 and arrows indicate the location of MSP and the subsequently designed qMSP primers and probe. In the lower part of the panels, dark circles indicate methylated CpGs, grey circles indicate partially methylated CpGs, and white circles indicate unmethylated CpGs. The MSP column on the right side of each panel (M, U/M and U) lists the methylation status as assessed by MSP. The box plots in the rightmost column visualize the differences in qMSP PMR values between cell lines that are unmethylated (U), partially methylated (U/M) and fully methylated (M) as assessed by the MSP analysis. In general, there is a good concordance between the qualitative and quantitative results, although this is only statistically significant for *DCLK1*.

Supplementary Figure 3. Receiver operating characteristics curves for fresh frozen and archival sample series. The panels depict the resulting area under the receiver operating characteristics curve based on the PMR values for individual biomarkers in the a) fresh frozen sample series and b) archival sample series. c) and d) show the overall performance of the biomarker panel in fresh frozen and archival sample series, respectively

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Supplementary tables

Supplementary Table 1: Overview of methylated genes previously identified in CCA

Gene symbol	Location	Function	Sensitivity	Specificity	Number of CCAs	Refs

			(%)	**(%)**	**analyzed**	
APC	5q21	Cell adhesion	26-46	100, 90	72-111	[1,2,3]
BCL2	18q21	Apoptosis	23	97	111	[1]
CACNA1G	17q22	Ion channel	4	94	111	[1]
CDH1	16q22	Cell adhesion	22-43	100, 90	15-111	[4,2,5,3,1]
CDKN2A ($p14^{ARF}$)	9p21	Cell cycle regulation	9- 38	100	51-111	[6,3,2,1]
CDKN2A ($p16^{INK4a}$)	9p21	Cell cycle regulation	14-77	100, 90	7-111	[7,8,1,4,2,6,5,3]
CDKN2B ($p15^{INK4b}$)	9p21	Cell cycle regulation	51	100	72	[3]
CHFR	12q24	Apoptosis	5, 17	100	23, 111	[1,5]
DAPK1	9q34	Apoptosis	3-40	100, 93	15-79	[4,9,5,3,2]
FHIT	3p14	Purine metabolism	42	89	19	[10]
GSTP1	11q13	Drug metabolism	6-18	100, 90	72-111	[1,2,3]
HOXA1	7p15.2	Development	90	95	111	[1]
IGF2	11p15.5	Cell growth and differentiation	23	89	111	[1]
MGMT	10q26	DNA repair	4-33	100	15-111	[1,4,2,3]

MINT1	22q11	Unknown	38, 41	100	79, 111	[1,2]
MINT12	22q11	Unknown	51	100	79	[2]
MINT2	22q11	Unknown	0, 7	100	79, 111	[1,2]
MINT25	22q11	Unknown	15	100	79	[2]
MINT31	22q11	Unknown	1, 15	100	79, 111	[1,2]
MINT32	22q11	Unknown	35	100	79	[2]
MLH1	3p22	DNA repair	13-47	100	15-72	[4,5,3]
NEUROG1	5q23	Cell differentiation	53	89	111	[1]
PTGS2	1q25	Biosynthesis in inflammation	5	100	79	[2]
PYCARD	16p11	Apoptosis	36	92	36	[11]
RARB	3p24	Cell growth and differentiation	14-18	100	72, 111	[1,3]
RARRES1	3q25	Membrane protein	22	97	111	[1]
RASSF1	3p21	Cell cycle regulation	28-73	50-100	13-111	[10,1,12,5,13,3]
RBP1	3q21	Retinol transport	14	100	111	[1]
RUNX3	1p36	Apoptosis	33, 78	100	23,111	[1,5]

SEMA3B	3p21	Apoptosis	100	100	15	[12]
SFN	1p36	Apoptosis	59	100	79	[2]
SFRP1	8p11	Cell growth and differentiation	63	91	41	[14]
SOCS3	17q25	Cytokine signaling	88	100	8	[15]
THBS1	15q15	Cell adhesion	2-11	100	79, 111	[1,2]
TIMP3	22q12	Cell adhesion	1-9	100	79-111	[1,2]
TMEFF2	2q32.3	Cell growth and differentiation	73	92	111	[1]
TP73	1p36	Cell cycle regulation	36	100	72	[3]
ZMYND10	3p21	Unknown	20	100	15	[12]

Genes are listed according to approved gene symbols (HUGO Gene Nomenclature Committee). Genes reported to be unmethylated are not included in the table.

Supplementary table 2: Gene lists from Figure 2

N=30 (Up-regulated in treated cell lines and down-regulated in ICC (Miller et al.))	
Gene symbol	**Description**
BDKRB1	Bradykinin receptor B1
BEX4	Brain expressed, X-linked 4
CALCOCO1	Calcium binding and coiled-coil domain 1
CDO1	Cysteine dioxygenase, type I
CRISPLD2	Cysteine-rich secretory protein LCCL domain containing 2
CSRP1	Cysteine and glycine-rich protein 1
CXCL14	Chemokine (C-X-C motif) ligand 14
DNAH3	Dynein, axonemal, heavy chain 3
DPYSL3	Dihydropyrimidinase-like 3
DUSP5	Dual specificity phosphatase 5
FKBP1B	FK506 binding protein 1B
GNG11	Guanine nucleotide binding protein (G protein), gamma 11
GPR124	G protein-coupled receptor 124

GREM1	Gremlin 1
ID3	Inhibitor of DNA binding 3, dominant negative helix-loop-helix protein
INPP5A	Inositol polyphosphate-5-phosphatase
ITPR1	Inositol 1,4,5-trisphosphate receptor, type 1
LHX6	LIM homeobox 6
LMCD1	LIM and cysteine-rich domains 1
MLLT11	Myeloid/lymphoid or mixed-lineage leukemia (trithorax homolog, Drosophila); translocated to, 11
MT2A	Metallothionein 2A
PLAT	Plasminogen activator, tissue
RASIP1	Ras interacting protein 1
SERPINF1	Serpin peptidase inhibitor, clade F, member 1
STXBP1	Syntaxin binding protein 1
TCF4	Transcription factor 4
TMOD1	Tropomodulin 1
TPM2	Tropomyosin 2
ZNF331	Zinc finger protein 331
ZSCAN18	Zinc finger and SCAN domain containing 18

N=8 (Up-regulated in treated cell lines and down-regulated in ICC (Obama et al.))	
Gene symbol	**Description**
FAM3B	Family with sequence similarity 3, member B
HABP4	Hyaluronan binding protein 4
LUM	Lumican
MT1F	Metallothionein 1F
MT1X	Metallothionein 1X
RNASE4	Ribonuclease, RNase A family, 4
SLC46A3	Solute carrier family 46, member 3
TCN2	Transcobalamin II

N=6 (Up-regulated in treated cell lines and down-regulated in ICC (Miller et al. and Obama et al.))	
Gene symbol	**Description**
ATF3	Activating transcription factor 3
CTGF	Connective tissue growth factor
EGR2	Early growth response 2
PRG1	p53-responsive gene 1

PTGDS	Prostaglandin D2 synthase 21kDa
TIMP3	TIMP metallopeptidase inhibitor 3

N=2 (Up-regulated in treated cell lines and down-regulated in ICC (Miller et al. and Obama et al.) and ECC (Miller et al.))

Gene symbol	Description
CLU	Clusterin
TCN1	Transcobalamin I

N=13 (Up-regulated in treated cell lines and down-regulated in ECC and ICC (Miller et al.))

Gene symbol	Description
ASRGL1	Asparaginase like 1
C7orf58	Chromosome 7 open reading frame 58
DCLK1	Doublecortin-like kinase 1
FHL1	Four and a half LIM domains 1
IL6	Interleukin 6
KLF9	Kruppel-like factor 9
MMP1	Matrix metallopeptidase 1

NAP1L2	Nucleosome assembly protein 1-like 2
NR4A3	Nuclear receptor subfamily 4, group A, member 3
PDE2A	Phosphodiesterase 2A
REEP1	Receptor accessory protein 1
SFRP1	Secreted frizzled-related protein 1
SYT11	Synaptotagmin XI

N=1 (Up-regulated in treated cell lines and down-regulated in ECC (Miller et al.))

Gene symbol	**Description**
FOLR1	Folate receptor 1

Abbreviations: ICC, intrahepatic cholangiocarcinoma; ECC, extrahepatic cholangiocarcinoma.

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Supplementary Table 3: Culturing conditions of cancer cell lines

Cell line	Medium	Additives
TFK-1	Roswell Park Memorial Institute (RPMI) 1640 medium*	Penicillin-Streptomycin-Glutamine
EGI-1	Dulbecco's Modified Eagle Medium (DMEM)*	Penicillin-Streptomycin-Glutamine
HuCCT1	Roswell Park Memorial Institute (RPMI) 1640 medium*	Penicillin-Streptomycin-Glutamine
SK-ChA-1	Minimum Essential Medium (MEM)*	Penicillin-Streptomycin-Glutamine
Mz-ChA-1	Roswell Park Memorial Institute (RPMI) 1640 medium*	Penicillin-Streptomycin-Glutamine
Mz-ChA-2	Minimum Essential Medium (MEM)*	Penicillin-Streptomycin-Glutamine
KMCU	Dulbecco's Modified Eagle Medium (DMEM)*	Penicillin-Streptomycin-Glutamine
KMBC	Dulbecco's Modified Eagle Medium (DMEM)*	Penicillin-Streptomycin-Glutamine and Horse Serum*
PaCa-2	Dulbecco's Modified Eagle Medium (DMEM)*	Penicillin-Streptomycin-Glutamine and MEM Non Essential Amino Ac
HPAFII	Minimum Essential Medium (MEM)*	Penicillin-Streptomycin-Glutamine
BxBc-3	Roswell Park Memorial Institute (RPMI) 1640 medium*	Penicillin-Streptomycin-Glutamine
AsPc-1	Roswell Park Memorial Institute (RPMI) 1640 medium*	Penicillin-Streptomycin-Glutamine
SW48	Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 (D-MEM/F12)*	Penicillin-Streptomycin-Glutamine
SW480	Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 (D-MEM/F12)*	Penicillin-Streptomycin-Glutamine
RKO	Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 (D-MEM/F12)*	Penicillin-Streptomycin-Glutamine
HCT15	Dulbecco's Modified Eagle Medium: Nutrient Mixture F-	Penicillin-Streptomycin-Glutamine

	12 (D-MEM/F12)*	
LS1034	Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 (D-MEM/F12)*	Penicillin-Streptomycin-Glutamine
HT29	Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 (D-MEM/F12)*	Penicillin-Streptomycin-Glutamine
HB8065	Minimum Essential Medium (MEM)*	Penicillin-Streptomycin-Glutamine
JHH-1	William's Medium E*	Penicillin-Streptomycin-Glutamine
JHH-4	Minimum Essential Medium (MEM)*	Penicillin-Streptomycin-Glutamine
JHH-5	William's Medium E*	Penicillin-Streptomycin-Glutamine
Panc-1	Dulbecco's Modified Eagle Medium (DMEM)*	Penicillin-Streptomycin-Glutamine
CFPAC-1	Iscove's Modified Dulbecco's Medium (IMDM)#	Penicillin-Streptomycin-Glutamine

Medium and additives were added according to requirements for each cell line.

*Gibco, Invitrogen, Carlsbad, CA, USA

#ATCC, Manassas, VA, USA

Supplementary Table 4: Cancer cell line genotypes

Cell line	AMEL	CSF1PO	D13S317	D16S539	D18S51	D19S433	D21S11	D2S1338	D3S1358	D5S818	D7S820	D8S1179	FGA	TH01	TPOX	vWA
KMBC	X	12	11	10	13	14, 15.2	29	17	15	12, 13	10, 12	10, 14	22	9.3	8, 11	16
KMCH-1	XY	11	10	9	14, 17	13, 14.2	30, 32.2	22, 25	16	10, 12	10, 12	10, 16	22, 23	9	8, 11	14, 18, 19
SK-ChA-1	X	12, 13	11, 12	9, 13	16	13.2, 14	28	25	15	11, 13	10	13, 14	25	6	8	14, 18
Mz-ChA-1	X	11	8, 10	11, 12	14	13, 15	28	16, 24	15	11	13, 14	13	20, 23	7	8	15, 19
Mz-ChA-2	X	12	12, 13	11	15	13, 16	28, 30	17, 26	18	10	11, 12	13	22, 24	8	8, 11	15, 17

Genotypes for non-commercial cell lines (listed here) were obtained using the AmpFLSTR Identifiler PCR Amplification Kit (Applied Biosystems). The size of the analyzed short tandem repeats (STR), analyzed by PCR and subsequent fragment analysis, are shown for each loci. Amelogenin (Amel) is a gender-determining locus

Supplementary Table 5: Primer sequences, fragment size, and PCR conditions

Primer set	Sense primer	Antisense primer	Frg. Size (bp)	Fragment location	An. Temp (°C)	$MgCl_2$ (mM)	El. Time (sec)	Accession number
ASRGL1_MSP_M	GAGATAGGTGCGCGTTAGTC	ACAACGATTCTACGCCTACG	91	+60 to +151	57	1.5	30	NM_001083926
ASRGL1_MSP_U	AGTGAGATAGGTGTGTGTTAGTT	ACAACAATTCTACACCTACACAC	94	+57 to +151	57	1.5	30	
ATF3_MSP_M	AGCGAGTACGTATATTTGGC	AAAACGAAACCGAAAACG	174	-221 to -47	53	1.5	30	NM_001040619
ATF3_MSP_U	AGTAGTGAGTATGTATATTTGGT	ACCAAAACAAAACCAAAAACA	180	-224 to -44	53	1.5	30	
BEX4_MSP_M	AGGGGTTGATTCGAAAGTTTC	TCTAACGCCAAAACGAAACA	132	-90 to +42	55	1.5	30	NM_001080425
BEX4_MSP_U	GATAGGGGTTGATTTGAAAGTTTT	AACTCTAACACCAAAACAAAACA	138	-93 to +45	55	1.5	30	
CALCOCO1_MSP_M	TACGTTTTTTAGGATGTCGC	CTTTTACCGCTACGTACTCG	116	-118 to -2	55	1.5	30	NM_020898
CALCOCO1_MSP_U	AATTATGTTTTTTAGGATGTTGT	CCCTTTTACCACTACATACTCAA	121	-121 to 0	55	1.5	30	
CDO1_MSP_M	TTGGGACGTCGGAGATAAC	GACCCTCGAAAAAAAAACGA	145	-153 to -8	53	1.5	30	NM_001801
CDO1_MSP_U	TTTTTGGGATGTTGGAGATAAT	AACCCTCAAAAAAAAAACAAAAC	148	-156 to -8	53	1.5	30	
CDO1_BS	TTTTTTTTGTTTAYGTTTTA	ACAAATCAAATTCAAATCT	350	-280 to +70	49	1.7	30	
CLU_MSP_M	TTTTTTTTATTGGAAGCG	AAAAAATACCGCGAAA	165	-147 to	52	2.4	30	NM_001831

	TC	AAC		+18				
	GGTTTTTTTTTATTGGAA	CCAAAAAATACCACAAA		-150 to				
CLU_MSP_U	GTGTT	AAACA	170	+20	52	2.4	30	
CRISPLD2_MS		ACTCAACGTACCGCCTC						
P_M	TTCGTTTATTCGGCGTTC	TT	172	-178 to -6	52	1.5	30	NM_031476
CRISPLD2_MS	TTTTTTGTTTATTTGGTG	AAAACTCAACATACCAC						
P_U	TTT	CTCTT	178	-181 to -3	52	1.5	30	
CSRP1_MSP_	ACGTGTAAGACGTTTTT	AACCCGACGATACTACC		-126 to				
M	CGC	CTC	147	+21	55	1.7	30	NM_004078
	GTATGTGTAAGATGTTT	AACCCAACAATACTACC		-128 to				
CSRP1_MSP_U	TTTGT	CTCCT	149	+21	56	1.5	30	
	TCGGAGCGTATAAAAGT	CTATCGACCGAAACGAC		-34 to				
CTGF_MSP_M	TTC	TAC	122	+88	56	2.5	30	NM_001901
	GTTTGGAGTGTATAAAA	CTATCAACCAAAACAAC		-36 to				
CTGF_MSP_U	GTTTT	TACCA	124	+88	56	2.5	30	
DCLK1_MSP_	GCGTTTTGTTAAGAAGG	ACGCGCTCCCTTTTCTTA		-127 to -				
M	GC	T	108	19	53	1.5	30	NM_004734
	GTGTTTTGTTAAGAAGG	ACACACTCCCTTTTCTTA		-127 to -				
DCLK1_MSP_U	GT	T	108	19	53	1.5	30	
	AAGATTATTTGTGGGGA	AACCTCTCTCTCCAAAA		-247 to				
DCLK1_BS	TTAGG	AAAAA	271	+24	57	1.5	30	
DUSP5_MSP_	GAGTGAGTTTTTTAGCG	ATAAATACCGTCCGTAA		-192 to				
M	AAGC	CGC	198	+6	52	1.5	30	NM_004419
DUSP5_MSP_	GAGTGAGTTTTTTAGTG	ATAAATACCATCCATAA		-192 to				
U	AAGT	CAC	198	+6	52	1.5	30	
	TATATGGGTAGCGACGT	TCGCCGAACTATTAATC						NM_001136
EGR2_MSP_M	TAC	AATTA	104	-108 to -4	52	2.0	30	177
	TTATATATGGGTAGTGA	CCCTCACCAAACTATTA						
EGR2_MSP_U	TGTTAT	ATCAATTA	110	-111 to -1	52	1.5	30	

FAM3B_MSP_M	GGGGAACGGGTTTATTTTTC	GCGACCAATCGAACAAAT	137	-120 to +17	53	1.5	30	NM_058186
FAM3B_MSP_U	GGGGAATGGGTTTATTTTTT	ACAACCAATCAAACAAAT	137	-120 to +17	53	1.5	30	
FHL1_MSP_M	TCGTGTAGTGGGTAGAGTTC	CTCCGCCGAACGATAAAT	165	-160 to +5	57	1.5	30	NM_001449
FHL1_MSP_U	TTTTTGTGTAGTGGGTAGAGTTT	CCCCTCCACCAAACAATAAAT	171	-163 to +8	57	1.5	30	
FKBP1B_MSP_M	GGTTCGTTAATAGTCGGGC	CTAAAATCGAAACCTACGCG	126	-158 to -32	55	2.0	30	NM_054033
FKBP1B_MSP_U	TTAGGTTTGTTAATAGTTGGGT	ACTAAAATCAAAACCTACACAAA	130	-161 to -31	52	1.5	30	
GNG11_MSP_M	TCGGATGTGATTTGGAAAC	CGCGAAAAACGACTAACT	112	-48 to +64	56	1.5	30	NM_004126
GNG11_MSP_U	ATTTGGATGTGATTTGGAAAT	CCCACAAAAAACAACTAAACT	116	-50 to +66	56	1.5	30	
GPR124_MSP_M	GGGTTTAGGTTTGGTCGC	CCGCTCCGTACCATAAATAA	119	-124 to -5	55	2.5	30	NM_032777
GPR124_MSP_U	AGAGGGTTTAGGTTTGGTTGT	CCACCACTCCATACCATAAATAA	125	-127 to -2	55	1.5	30	
GREM1_MSP_M	AGTAGATAAAGAGGCGAGGC	AAATACCGACGACAAAACG	172	-198 to -26	53	1.5	30	NM_013372
GREM1_MSP_U	GGGAGTAGATAAAGAGGTGAGGT	AAATACCAACAACAAAACACAA	175	-201 to -26	53	1.5	30	
HABP4_MSP_M	CGTGACGTGATAGTAGCGGTC	CTATCCGACCCCTACCGAC	149	-115 to +34	58	1.5	30	NM_014282
HABP4_MSP_U	GTGTGATGTGATAGTAGTTGGTT	CCTATCCAACCCCTACCAAC	151	-116 to +35	59	1.5	30	
ID3_MSP_M	TTCGGAGGAGTTGTGGTTTCGG	CGCTAATACCGAAAAAA	173	-32 to	55	1.5	30	NM_002167

	TC	AACG		+141				
	GATTTTGGAGGAGTTGT	CACTAATACCAAAAAAA		-35 to				
ID3_MSP_U	GGTTT	AACAAAC	176	+141	55	1.5	30	
INPP5A_MSP_	TTAGCGGATTTAATGGT	TAACCGAAACTCCGACC		-20 to				
M	TGC	TC	113	+93	50	1.5	30	NM_005539
INPP5A_MSP_	TTAGTGGATTTAATGGT	TAACCAAAACTCCAACC		-20 to				
U	TGT	TC	113	+93	50	1.5	30	
	ATTTAGGGTTTAGTTCG	ACACTTTAAAACGACTC		-146 to				
ITPR1_MSP_M	GGC	CGAA	148	+2	55	2.5	30	NM_002222
	TTTATTTAGGGTTTAGTT	ACTACACTTTAAAACAA		-149 to				
ITPR1_MSP_U	TGGGT	CTCCAAA	154	+5	55	2.0	30	
	TGCGGTTGTGGTTTTTTT	CCGAAACGACGTTCTCA		-69 to				
LHX6_MSP_M	C	T	100	+31	54	1.5	30	NM_014368
	TATTGTGGTTGTGGTTT	ACACCAAAACAACATTC		-72 to				
LHX6_MSP_U	TTTTT	TCAT	106	+34	54	1.5	30	
LMCD1_MSP_	GGTAGTCGGCGTTTAGT	CGCAACTAAACCGCTTT		-176 to -				
M	TTC	AAT	165	11	55	1.5	30	NM_014583
LMCD1_MSP_	TAGGGTAGTTGGTGTTT	AAACACAACTAAACCAC						
U	AGTTTT	TTTAAT	171	-179 to -8	55	1.5	30	
MLLT11_MSP_	TTTTTCGGGTTAGTTTTG	AACCGAACGAATTTCGT						
M	C	AAT	110	-118 to -8	51	1.8	30	NM_006818
MLLT11_MSP_	GGGTTTTTTGGGTTAGT	CCAAACCAAACAAATTT						
U	TTTGT	CATAAT	116	-121 to -5	52	1.5	30	
	GTTTAGGGGATTTTGCG	ACAACCGACCGCTACTT		-110 to				
MT1F_MSP_M	TTC	TAA	147	+37	55	1.5	30	NM_005949
	GTTTAGGGGATTTTGTG	ACAACCAACCACTACTT		-110 to				
MT1F_MSP_U	TTT	TAA	147	+37	55	1.5	30	
	GGTTTACGGGTTGTTGT	AAAAACCGACGACTCTC						
MT1X_MSP_M	ATTC	TTT	129	-136 to -7	55	1.5	30	NM_005952

	GGGTTTATGGGTTGTTG	CAAAAACCAACAACTCT						
MT1X_MSP_U	TATTT	CTTT	131	-137 to -6	55	1.5	30	
MT2A_MSP_	GTGTGTAGAGTCGGGT	AAAACCGAAACGAATAC		-108 to -				
M	GC	AAAA	132	240	55	1.5	30	NM_005953
	GTGTGTAGAGTTGGGT	AAAACCAAAACAAATAC		-108 to -				
MT2A_MSP_U	GT	AAAA	132	240	55	1.5	30	
NAP1L2_MSP_	GCGTAATTATATTGCGG	TACGTTAACCGATCCTA		+8 to				
M	TATC	CAA	116	+124	56	1.5	30	NM_021963
NAP1L2_MSP_	GTTGTGTAATTATATTG	AACTACATTAACCAATC		+5 to				
U	TGGTATT	CTACAA	122	+127	56	1.5	30	
NR4A3_MSP_	TTTTCGTATACGCGGAA	TCGACACGTCATTTATA		-126 to				
M	TC	CCAC	142	+16	52	1.5	30	NM_173198
NR4A3_MSP_	TTTTTTTTGTATATGTGG	CTCTCAACACATCATTTA		-129 to				
U	AATT	TACCAC	148	+19	52	1.5	30	
PDE2A_MSP_	ATTAGGCGAAGTTGTCG	CGACTCGTCCGACTTAA		+10 to				NM_001143
M	C	AA	161	+171	53	1.8	30	839
PDE2A_MSP_	GGATTAGGTGAAGTTGT	AACAACTCATCCAACTT		+8 to				
U	TGT	AAAA	165	+173	53	1.8	30	
REEP1_MSP_	GGACGCGTTCGTTTTTA	AACCGCGACACGTTCTA		-162 to -				NM_001164
M	GTC	AC	149	13	55	2.5	30	732
	GTAGGATGTGTTTGTTT	AACCACAACACATTCTA		-165 to -				
REEP1_MSP_U	TTAGTT	ACAAC	152	13	55	2.5	30	
RNASE4_MSP_	TAAATTTCGGACGAGTT	TCGCGAAACAATTTATA		-143 to -				
M	TTC	TTTC	101	42	53	2.5	30	NM_002937
RNASE4_MSP_	GTTTAAATTTTGGATGA	CCATCACAAAACAATTT		-146 to -				
U	GTTTTT	ATATTTC	107	39	53	1.5	30	
SFRP1_MSP_	TAGTAAATCGAATTCGT	TACGCGAAACTCCTACG		-138 to				
M	TCGC	AC	141	+3	45	1.5	30	NM_003012
SFRP1_MSP_U	TTTTAGTAAATTGAATTT	TACACAAAACTCCTACA	144	-141 to	45	1.5	30	

	GTTTGT	ACCAA		+3				
SLC46A3_MSP	GTTGAGTGGTTGTTCGG	CCCGACTCTCCTACGAT						
_M	TC	TAA	151	-152 to -1	57	1.5	30	NM_181785
SLC46A3_MSP	GTGTTGAGTGGTTGTTT	TACCCAACTCTCCTACA		-154 to				
_U	GGTT	ATTAA	155	+1	58	1.5	30	
SYT11_MSP_	CGTTTTGGAATTATAGC	TTCCGAATAATCCTCGA		-222 to -				
M	GC	AA	158	64	50	1.8	30	NM_152280
	TTTTGTTTTGGAATTATA	CTCTTCCAAATAATCCTC		-225 to -				
SYT11_MSP_U	GTGT	AAAA	164	61	50	1.8	30	
TCF4_MSP_M	GAATTTGTAATTTCGTG	AAAAAAAACTCTCCGTA		+322 to				NM_001083
*	CGTTTC	CACCG	258	+580	57	1.5	60	962
	TGAATTTGTAATTTTGT	AAAAAAAACTCTCCATA		+321 to				
TCF4_MSP_U*	GTGTTTTG	CACCACC	259	+580	57	1.5	60	
	ATCGTCGGGGTTTTTTT	AACAAAAACACGACCCG						NM_001145
TPM2_MSP_M	AGTC	AC	152	-156 to -4	61	1.5	30	822
	GTATTGTTGGGGTTTTT	AAACAAAAACACAACCC						
TPM2_MSP_U	TTAGTT	AACC	155	-158 to -3	61	1.5	30	
ZNF331_MSP_	GGTAGGACGTTTTTAGG	ATACAACTCTACACGAC		-120 to				
M	GTC	GCA	143	+23	55	1.7	30	NM_018555
ZNF331_MSP_	TAAGGTAGGATGTTTTT	AACATACAACTCTACAC		-120 to				
U	AGGGTT	AACACA	143	+23	55	1.5	30	
ZSCAN18_MSP	GTTTAAAATGACGTAGG	AATACCGCGAAACTATA		-52 to				
_M	CGTC	CCG	131	+79	55	1.8	30	NM_023926
ZSCAN18_MSP	GGTGTTTAAAATGATGT	ACAATACCACAAAACTA		-55 to				
_U	AGGTGTT	TACCAC	131	+79	55	1.5	30	
	TTTTGGTTGTTAGGGGT	ACCCACCTACTACRCAA		-106 to				
ZSCAN18_BS	TTATT	CTAC	302	+196	59	1.5	30	

From hg18 to hg19, the transcription start point of *DCLK1* NM_004734 was moved 50 bp upstream. The MSP primers were originally designed to be located -177 to -69 relative to the

transcription start site. *Primer sequences and amplification conditions were obtained from Kim and colleagues.[16]