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APPENDICES

APPENDIX A
SAMPLE SIZE

Sample size

$$n = \frac{Z^2_{\alpha/2} P(1-P)}{d^2}$$

n	=	sample size
$Z^2_{\alpha/2}$	=	A standard normal random variable = 1.96
P	=	Prevalence of methylation = 0.06*
d	=	Error in estimation: P = $\pm$0.05 (2 directions)

* Prevalence derived from the frequency of *Fas* alterations detected in 36 cases of the pilot study = 0.06

$$\mathbf{n = \frac{(1.96)^2\,(0.06)(1-0.06)}{(0.05)^2} = 86.7 \approx 87 \text{ samples}}$$

APPENDIX B
CLINICOPATHOLOGICAL DATA

Table B-1 Clinicopathological data of CCA patients (n=102).

CODE	Gross type	Differentiated type	Age	Sex	Survival (weeks)	Status
N044	MF	Less	73	F	21.71429	Died
N069	MF	Less	40	M	26.57143	Died
N082	MF	Well	43	F	12.28571	Died
N104	PI	Well	55	M	12	Died
N125	PI	Well	40	M	3.285714	Died
N128	PI	Well	49	M	27.57143	Died
P010	ID	Well	53	M	85.14286	Died
P048	MF	Well	73	M	29.85714	Died
P055	MF	Well	64	M	7.857143	Died
P059	PI	Well	46	F	14.14286	Died
P060	MF	Well	64	F	201	Died
P068	PI	Well	69	M	92	Died
P077	MF	Less	43	M	12.85714	Died
P086	MF	Well	38	M	24.42857	Died
P091	PI	Well	54	M	356.2857	Alive
P092	MF	Well	55	M	10.42857	Died
P126	MF	Well	51	M	163.7143	Died
P127	MF	Less	62	M	25.85714	Died
P145	MF	Less	43	M	29.28571	Died
P147	MF	Well	61	F	39.28571	Died
P152	MF	Less	56	F	21.28571	Died
Q044	MF	Less	39	M	75.85714	Died
Q045	MF	Less	58	M	30.14286	Died
Q053	PI	Less	63	M	22.42857	Died
Q063	MF	Less	43	M	41.71429	Died
Q093	MF	Less	51	M	25	Died
Q095	MF	Less	45	F	12	Died
Q102	PI	Less	41	M	4.857143	Died
R004	MF	Less	67	M	70.85714	Died
R009	MF	Less	74	M	17.57143	Died
R023	MF	Well	51	M	48.85714	Died

Table B-1 Clinicopathological data of CCA patients (n=102) (cont.).

CODE	Gross type	Differentiated type	Age	Sex	Survival (weeks)	Status
R052	MF	Well	56	F	125.5714	Died
R054	PI	Well	55	M	88	Died
R078	MF	Less	55	M	10.71429	Died
R081	PI	Well	60	M	12.71429	Died
R086	MF	Well	68	M	40.14286	Died
R087	ID	Well	56	M	56.28571	Died
R093	PI	Well	44	M	7	Died
R100	PI	Well	49	F	35.85714	Died
R103	MF	Well	39	M	6	Died
R105	MF	Less	56	M	28.85714	Died
R106	MF	Well	48	F	15	Died
R145	MF	Well	62	F	235.4286	Alive
R156	PI	Less	47	F	8.857143	Died
T005	MF	Well	63	M	73.57143	Died
T046	PI	Well	43	M	73	Died
T049	MF	Well	67	F	71	Died
T064	MF	Well	66	M	8.428571	Died
T092	MF	Less	67	M	15.57143	Died
T098	MF	Well	76	M	12.85714	Died
U005	MF	Well	54	M	44.14286	Died
U021	MF	Well	66	M	97	Died
U022	MF	Well	53	F	19.57143	Died
U027	MF	Well	66	F	21.14286	Died
U030	MF	Well	49	M	14.14286	Died
U034	MF	Less	59	F	37.14286	Died
U035	PI	Well	48	M	10.42857	Died
U039	MF	Well	43	F	34.71429	Died
U041	PI	Well	59	M	17.57143	Died
U044	MF	Well	53	M	6.428571	Died
U059	MF	Well	59	M	8.571429	Died
U064	MF	Well	56	F	15	Died

Table B-1 Clinicopathological data of CCA patients (n=102) (cont.).

CODE	Gross type	Differentiated type	Age	Sex	Survival (weeks)	Status
U071	ID	Well	59	M	13.85714	Died
U077	MF	Well	38	M	30	Died
U081	MF	Well	45	F	24.71429	Died
U090	MF	Well	71	F	62.14286	Died
U095	MF	Well	62	F	5	Died
U110	MF	Less	60	M	104.1429	Died
W008	ID	Well	39	M	123.4286	Alive
W010	ID	Well	38	M	42.28571	Died
W011	MF	Well	62	M	123	Alive
W012	PI	Well	61	F	21.28571	Died
W016	MF	Well	62	F	17.57143	Died
W022	MF	Well	65	M	21.71429	Died
W025	MF	Well	48	F	69.71429	Died
W027	MF	Well	54	F	48.85714	Died
W028	PI	Well	54	M	16.28571	Died
W036	MF	Less	52	M	114.2857	Alive
W039	ID	Well	66	M	9.142857	Died
W046	PI	Well	45	F	111.2857	Alive
W048	MF	Well	45	F	110.4286	Alive
W060	MF	Well	54	M	19.14286	Died
W068	MF	Well	56	M	107	Alive
W093	PI	Well	58	F	36.71429	Died
W097	PI	Well	55	F	50.28571	Died
W100	ID	Less	55	M	14.42857	Died
W108	PI	Well	64	M	52.14286	Died
W110	PI	Well	70	M	14.57143	Died
W111	MF	Well	56	M	42.42857	Died
W128	MF	Well	57	F	38.14286	Died
W132	PI	Well	44	M	20.14286	Died
W147	MF	Well	55	M	58.28571	Died
W149	ID	Well	57	M	9.285714	Died

Table B-1 Clinicopathological data of CCA patients (n=102) (cont.).

CODE	Gross type	Differentiated type	Age	Sex	Survival (weeks)	Status
W164	MF	Well	43	F	86.28571	Alive
W169	MF	Less	75	M	5.142857	Died
W175	ID	Well	40	F	81.28571	Alive
W176	ID	Well	62	M	81.14286	Alive
W183	MF	Less	38	F	12.57143	Died
W184	ID	No data	59	F	79	Alive
W187	PI	No data	69	M	5	Died
W189	PI	No data	49	M	23.57143	Died
W196	MF	Well	53	M	19.14286	Died

APPENDIX C
CHEMICALS AND REAGENTS

Table C-1 Chemicals and reagents.

Suppliers	Chemicals and reagents
Bioline, Randolph, MA	25 bp DNA ladder
Qiagen, Valencia, CA	FlexiGene DNA kit
	DNeasy Tissue Kit
Gibco-BRL, Ontario, Canada	RPMI 1640
	HAMF-12
Promega Corporation, Madison, WI	Wizard DNA Purification resin
	Agarose
New England Biolabs, Ipswich, MA	SssI methyltransferase enzyme
	S-adenosylmethionine
	Fnu4HI$^{(M)}$
	BsiEI$^{(M)}$
	Hpy118III$^{(M)}$
Sigma, St. Louis, MO	Sodium bisulfate
	Hydroquinone
	Diaminobenzidine (DAB)
Vector Laboratories, Peterborough, UK	2.5% normal horse serum
	ImmPRESS Universal Antibody Polymer Detection Kit
R&D Systems Europe Ltd, Abingdon, UK	Anti-human OBCAM: MAB27771
Santa Cruz Biotechnology Inc. Heidelberg, Germany	DcR1 (L19): sc-73890
Abcam, Cambridge, UK	Ethidium bromide
Pharmacia Biotech, Uppsala, Sweden	Deoxynucleoside triphosphate
	Ethylenediamine tetraacetic acid
	Tween-20

Table C-1 Chemicals and reagents (cont.).

Suppliers	Chemicals and reagents
BDH Laboratories supplies, UK	Hydrochloric acid
	Magnesium chloride
	Sodium chloride
Merk, Germany	Ethanol absolute
Pioneer Research Chemicals Limited, Essex, UK	Haematozylin
Fisher Scientific UK Ltd, Leicestershire, UK	Permount
	Xylene
Borhringer Mannheim, Germany	Proteinase K
Qiagen, West Sussex, UK	HotStarTaq DNA Polymerase and HotStarTaq Master Mix Kit
	EpiTect® Bisulfite Kit
Cambridge Bioscience , Cambridge, UK	EZ-96 DNA Methylation-Gold™ Kit (Zymo research)
Biotage, Uppsala, Sweden	PyroGold SQA reagent kit
	Binding Buffer (pyrosequencing)
	Annealing Buffer (pyrosequencing)
GE Healthcare, Amersham, UK	Streptavidin Sepharose HP coated beads

APPENDIX D

REAGENTS FOR MSP ASSAY

Reagents for MSP assay

1. 3 M Sodium acetate pH 5.2

Sodium acetate	408.1	g
Deionized water	750	ml

Adjusted pH to 5.2, then adjusted volume to 1000 ml. The solution was mixed and sterilized by autoclave at 121 °C, 15 pound/inch^2 for 20 min, and stored at room temperature.

2. 3 M Sodium bisulfite pH 5.0

Sodium bisulfite	18.8	g

Deionized water was added approximately 30 ml and mixed until Sodium bisulfite dissolved. Adjusted pH to 5.0, then adjusted volume to 50 ml. The solution was mixed and sterilized by 0.2 μM filter, and stored in 1 ml aliquots with light protection at -20 °C. The reagent should be used up to a month and freeze-thaw cycle is not allowed.

3. 10 Mm Hydroquinone

Hydroquinone	0.0101	g
Sterile deionized water	10	ml

The solution was mixed thoroughly and prepared just prior to use.

4. Column wash solution (80% Isopropanol)

Isopropanol	800	ml

Sterile deionized water was added to 1000 ml and stored at 4 °C.

5. 10X Methylase buffer

5 M NaCl	10	ml
0.5 M EDTA pH 7.9	20	ml

Deionized water was added to 100 ml. the solution was mixed and sterilized by 0.2 µM filter and stored at -20 °C.

6. 100 mM 2-mercaptoethanol

14.2 M 2-mercaptoethanol	70.126 µl

Deionized water was added to 100 ml. the solution was mixed and sterilized by 0.2 µM filter and stored at -20 °C.

7. 10X PCR buffer

10 M Ammonium sulfate	8.3	ml
1 M Tris-HCl pH 8.8	33.5	ml

Deionized water was added to 100 ml. the solution was mixed and sterilized by autoclave and stored in 1 ml aliquots at -20 °C.

APPENDIX E

SUPPLEMENTARY DATA FOR MSP RESULTS

Table E-1 Methylation frequencies and statistic difference in CCA and adjacent normal tissues.

CpG-island	% Methylation (n_{pos}/n_{tested})		*P*-value*
	CCA	Adjacent normal	
14-3-3σ	81.4 (83/102)	75.9 (22/29)	NS
OPCML	72.5 (74/102)	0 (0/29)	<0.001
SFRP-1	63.7 (65/102)	3.4 (1/29)	<0.001
HIC1	38.2 (39/102)	0 (0/29)	<0.001
PTEN	35.3 (36/102)	6.9 (2/29)	0.003
DcR1	28.4 (29/102)	3.4 (1/29)	0.005
MINT25	15.7 (16/102)	0 (0/29)	0.014
P16	15.7 (16/102)	0 (0/29)	0.014
RASSF1A	14.7 (15/102)	3.4 (1/29)	NS
BLU	10.8 (11/102)	13.8 (4/29)	NS
DAPK	9.8 (10/102)	3.4 (1/29)	NS
CASP8	1.9 (2/102)	13.8 (4/29)	0.022
FAS	0.98 (1/102)	0 (0/29)	NS
MGMT	0.98 (1/102)	0 (0/29)	NS
P73	0.98 (1/102)	0 (0/29)	NS
APAF-1	0 (0/102)	0 (0/29)	NS
BRCA1	0 (0/102)	0 (0/29)	NS
DcR2	0 (0/102)	0 (0/102)	NS
DR4	0 (0/102)	0 (0/102)	NS
DR5	0 (0/102)	0 (0/102)	NS
FancF	0 (0/102)	0 (0/29)	NS
GSTP1	0 (0/102)	10.3 (3/29)	0.010
P21	0 (0/102)	0 (0/29)	NS
SOCS-3	0 (0/102)	3.4 (1/29)	NS
Survivin	0 (0/102)	0 (0/29)	NS
TMS1	0 (0/102)	0 (0/29)	NS

NS = no statistical significance, * Mann-Whitney U test

APPENDIX F
SUPPLEMENTARY DATA FOR PYROSEQUENCING AND COBRA

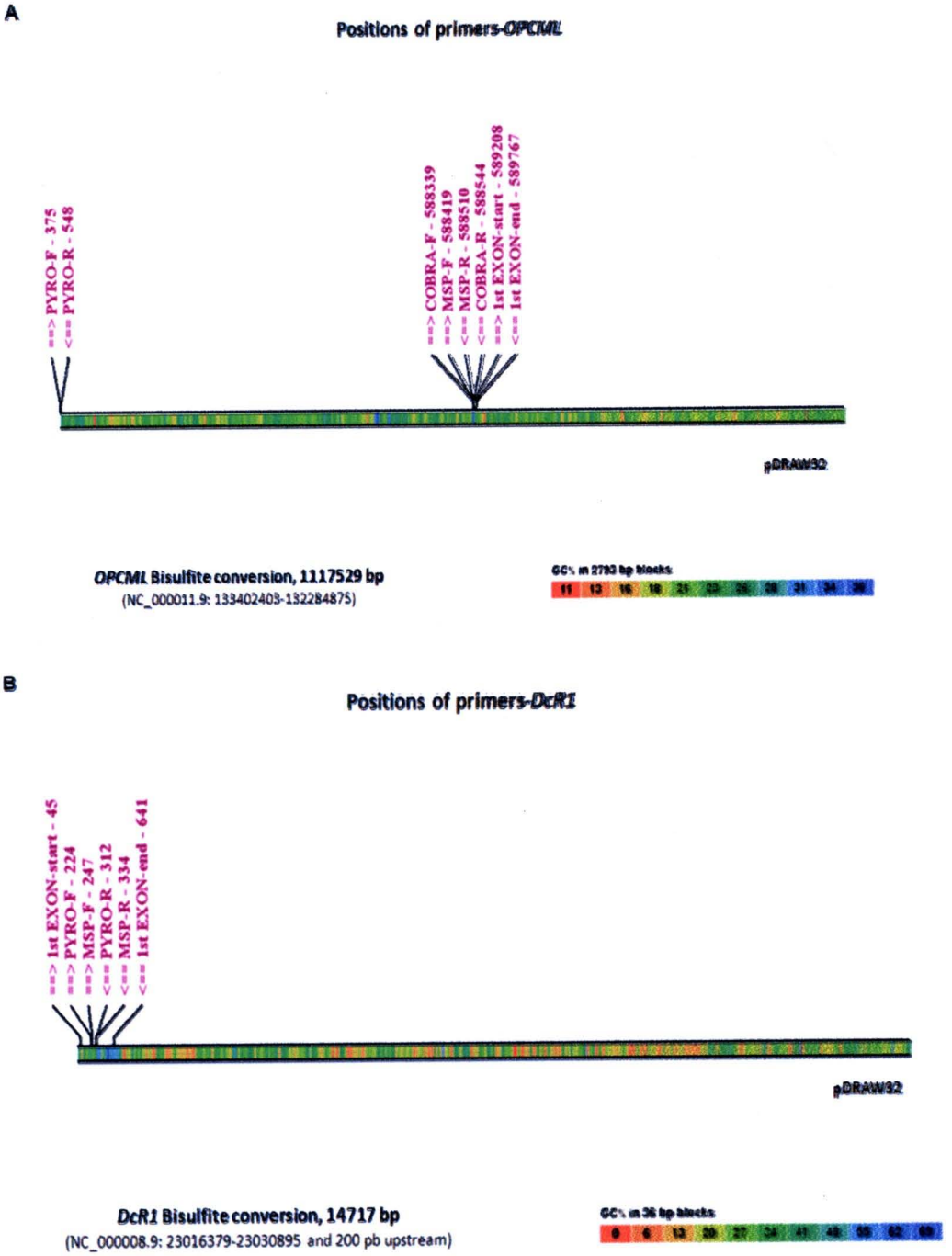


Figure F-1 The schematics of specific primers for MSP, pyrosequencing and COBRA in *OPCML* (A) and *DcR1* (B).

Table F-1 The association between methylation of *DcR1* and *OPCML* (pyrosequencing) and survival time of CCA patients.

Gene	*P*- value*
DCR1	
CpG1	0.080
CpG2	0.116
CpG3	0.146
CpG4	0.121
Average	0.107
OPCML	
CpG1	0.433
CpG2	0.992
CpG3	0.657
CpG4	0.351
CpG5	0.247
CpG6	0.987
Average	0.555

*Univariate survival analysis by Cox-regression

Table F-2 The cut-off of methylation of *DcR1* and *OPCML* in each CpG site and average by pyrosequencing and methylation frequency in CCA.

Gene	Methylation cut-off (%)	Methylation frequency (%)
DCR1		
CpG1	34.99753	7.1
CpG2	49.12737	5.1
CpG3	12.2982	15.3
CpG4	19.42853	15.3
Average	28.40687	10.2
OPCML		
CpG1	11.3125	56.86
CpG2	32.625	46.08
CpG3	12.4375	53.92
CpG4	9.25	57.84
CpG5	9.5	61.76
CpG6	13.6875	46.08
Average	13.59375	57.84

Cut-off = 1.5(IQR) + Q_3
IQR: interquartile range, Q3: 75% percentile or third quartile

APPENDIX G

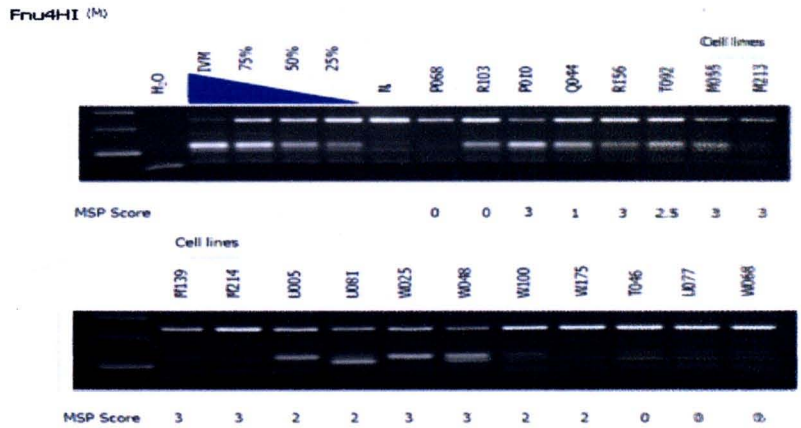
VALIDATION RESULTS OF METHYALATION STUDY IN *OPCML*, *SFRP1*, *HIC1* and *DcR1*

Table G-1 Validation of methylation status in highly methylated CpG-islands comparing between MSP and pyrosequencing or COBRA.

CpG-island	Validated result (%) ($n_{validated}/n_{tested}$)	*P*-value*	Method
OPCML	80 (12/15)	0.025	COBRA
	62.8 (64/102)	0.784	Pyrosequencing
SFRP-1	73.3 (11/15)	0.171	COBRA
HIC1	71.4 (10/14)	0.040	COBRA
DcR1	82.7 (81/98)	<0.001	Pyrosequencing

*Chi-square test, NS = No statistic significance

A



B

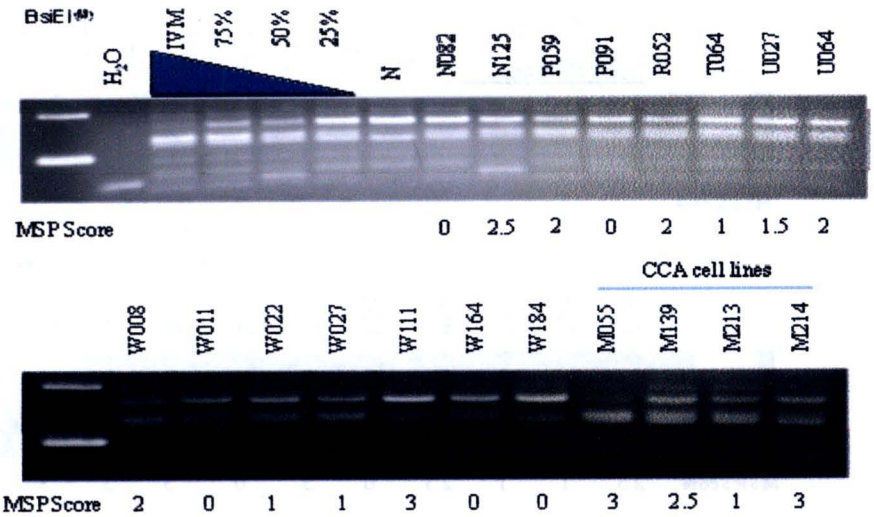
Sample	COBRA	MSP
P068	-	-
R103	+	-
P010	+	+
Q044	+	+
R156	+	+
T092	+	+
U005	+	+
U081	+	+
W025	+	+
W048	+	+
W100	-	+
W175	-	+
T046	-	-
U077	-	-
W068	-	-
KKU-M055	+	+
KKU-M139	-	+
KKU-M213	+	+
KKU-M214	+	+

+ positive methylation

- negative methylation

Figure G-1 COBRA study of *OPCML* in CCA, CCA cell line and controls. Results of restriction enzyme digestion (A) and COBRA compared to MSP (B).

A



B

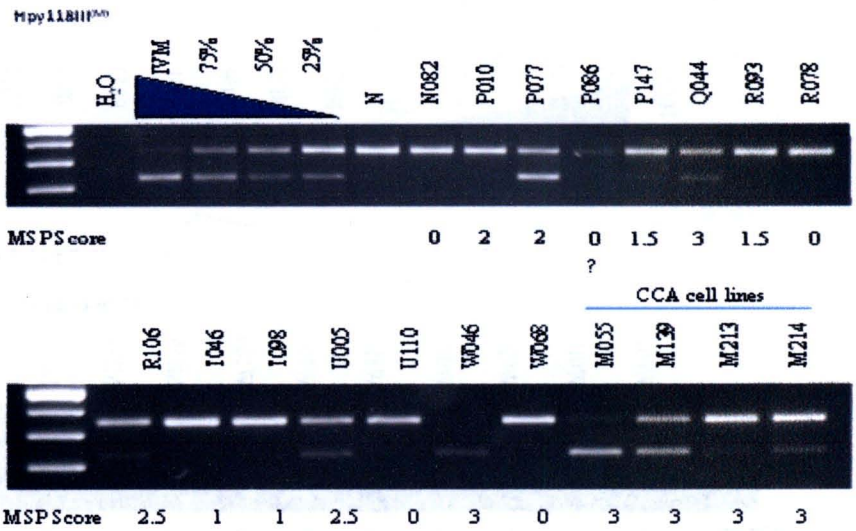
Sample	COBRA	MSP
N082	+	-
N125	+	+
P059	+	+
P091	+	-
R052	+	+
T064	+	+
U027	+	+
U064	+	+
W008	+	+
W011	-	-
W022	+	+
W027	+	+
W111	-	+
W164	+	-
W184	-	-
KKU-M055	+	+
KKU-M139	+	+
KKU-M213	+	+
KKU-M214	+	+

+ positive methylation

- negative methylation

Figure G-2 COBRA study of *SFRP-1* in CCA, CCA cell line and controls. Results of restriction enzyme digestion (A) and COBRA compared to MSP (B).

A



B

Sample	COBRA	MSP
N082	-	-
P010	-	+
P077	+	+
P086	Low PCR product?	-
P147	+/-	+
Q044	+	+
R093	-	+
R078	-	-
R106	+	+
I046	-	+
I098	-	+
U005	+	+
U110	-	-
W046	+	+
W068	-	-
KKU-M055	+	+
KKU-M139	+	+
KKU-M213	+/-	+
KKU-M214	+	+

+ positive methylation

- negative methylation

Figure G-3 COBRA study of *HIC1* in CCA, CCA cell line and controls. Results of restriction enzyme digestion (A) and COBRA compared to MSP (B).

APPENDIX H
REAGENTS FOR IMMUNOHISTOCHEMISTRY

Reagents for Immunohistochemistry

1. 1 mM EDTA solution pH 9.0

EDTA	0.37	g
Distilled water	900	ml

Adjusted pH to 9.0, then adjusted volume to 1000 ml. 0.5 ml of Tween-20 was added and mixed. Store this solution at room temperature for 3 months or at 4 °C for longer storage.

2. 0.6% Hydrogen peroxide solution

30% Hydrogen peroxide	6	ml
Distilled water	300	ml

Mixed well and prepared just prior to use.

3. 10X Phosphate buffered-saline (PBS, pH 7.4)

NaH_2PO_4 (anhydrous)	10.9	g
NaH2PO4 (anhydrous)	3.2	g
NaCl	90	g

Added distilled water approximately 900 ml, then mixed to dissolve and adjusted pH to 7.4. Adjusted volume to 1000 ml and stored the solution at room temperature.

4. 1X PBS

10X PBS	100	ml
Distilled water	900	ml

Mixed well and stored at room temperature until use.

5. Antibody diluent

1X PBS	100	ml
Tween-20	50	µl
Sodium azide	0.05	g

Mixed well and stored at 4 °C.

APPENDIX I

IMMUNOHISTOCHEMISTRY AND METHYLATION RESULTS OF OPCML AND DcR1

Table I-1 Protein expression by immunohistochemistry (IHC) and methylation status of OPCML and DcR1 by MSP in CCA samples.

CCA	OPCML		DcR1	
	Methylation	**IHC**	**Methylation**	**IHC**
N044	+	neg	+	1+
N069	+	ND	-	ND
N082	+	2+	-	neg
N104	-	1+	-	neg,1+
N125	+	neg	-	1+,2+
N128	+	1+	+	1+
P010	+	neg 1+	-	neg
P048	+	neg	-	neg
P055	-	neg, 1+	-	1+
P059	+	1+	-	2+3+
P060	+	1+	+	ND
P068	-	ND	+	2+
P077	+	1+	-	1+
P086	+	neg, 1+	+	neg
P091	-	1+	-	2+
P092	-	neg	-	3+
P126	+	1+	-	neg
P127	-	neg	-	neg
P145	+	neg	-	1+
P147	+	1+	-	1+,2+
P152	+	1+	+	1+
Q044	+	lymph node	-	ND
Q045	+	1+	-	neg
Q053	+	neg	+	neg
Q063	+	2+	+	1+,2+3+
Q093	+	neg, 1+	-	2+
Q095	+	2+	+	neg
Q102	+	1+	-	3+
R004	+	ND	+	ND
R009	-	ND	-	ND
R023	+	neg, 1+	+	neg

Methylation: + = positive, - = negative
IHC intensity: neg = negative, 1+ = weakly, 2+ = moderately, 3+ = strongly positive
ND = not done

Table I-1 Protein expression by immunohistochemistry (IHC) and methylation status of OPCML and DcR1 by MSP in CCA samples (cont.).

CCA	OPCML		DcR1	
	Methylation	**IHC**	**Methylation**	**IHC**
R052	-	ND	+	ND
R054	+	neg	-	neg
R078	+	1+	+	neg
R081	+	2+	-	3+
R086	+	1+	-	neg,1+
R087	+	1+	-	1+
R093	+	1+	-	neg
R100	+	2+	+	1+,2+
R103	-	neg, 1+	-	ND
R105	+	1+	-	neg
R106	+	2+	-	2+
R145	+	1+	+	neg
R156	+	1+	-	neg,1+
T005	+	1+	-	neg,1+
T046	-	neg	+	2+
T049	+	neg	+	neg
T064	+	1+	+	neg
T092	+	neg	-	3+
T098	+	neg	+	neg
U005	+	neg	-	neg
U021	+	2+, 1+	+	1+
U022	+	neg	-	neg
U027	+	ND	-	ND
U030	-	1+,2+	-	neg
U034	+	1+	+	2+
U035	-	neg	-	2+
U039	-	2+	-	neg
U041	-	neg, 1+	-	1+
U044	+	1+	-	1+
U059	+	neg	-	neg

Methylation: + = positive, - = negative
IHC intensity: neg = negative, 1+ = weakly, 2+ = moderately, 3+ = strongly positive
ND = not done

Table I-1 Protein expression by immunohistochemistry (IHC) and methylation status of OPCML and DcR1 by MSP in CCA samples (cont.).

CCA	OPCML		DcR1	
	Methylation	**IHC**	**Methylation**	**IHC**
U064	-	1+, neg	+	neg
U071	-	1+	+	neg
U077	-	neg, 1+	-	neg
U081	+	neg, 1+	-	2+
U090	-	neg, 1+	-	neg
U095	-	neg, 1+	-	neg
U110	-	1+	-	neg
W008	+	1+	-	1+
W010	+	neg	-	neg
W011	-	neg	-	neg
W012	+	ND	-	neg
W016	+	1+, neg	-	neg
W022	+	neg 1+	-	1+
W025	+	neg	+	neg
W027	+	2+	-	3+
W028	+	ND	-	ND
W036	+	1+	+	2+
W039	-	neg, 1+	-	1+
W046	+	1+	+	neg
W048	+	neg, 1+	+	neg
W060	-	1+	-	neg
W068	-	1+	-	1+
W093	+	neg, 1+	+	neg
W097	-	neg, 1+	-	neg
W100	+	neg	-	1+
W108	+	neg, 1+	-	neg
W110	+	ND	-	ND
W111	-	neg	-	1+
W128	+	1+	-	neg

Methylation: + = positive, - = negative
IHC intensity: neg = negative, 1+ = weakly, 2+ = moderately, 3+ = strongly positive
ND = not done

Table I-1 Protein expression by immunohistochemistry (IHC) and methylation status of OPCML and DcR1 by MSP in CCA samples (cont.).

CCA	OPCML		DcR1	
	Methylation	**IHC**	**Methylation**	**IHC**
W132	+	neg, 1+	-	3+
W147	+	neg	-	neg
W149	+	1+	-	neg
W164	-	neg	-	neg
W169	+	1+	-	1+
W175	+	neg, 1+	-	1+
W176	+	1+	+	neg
W183	+	neg 1+	-	neg
W184	-	neg, 1+	-	neg
W187	+	1+	-	neg
W189	+	1+	-	neg
W196	+	neg	-	neg

Methylation: + = positive, - = negative
IHC intensity: neg = negative, 1+ = weakly, 2+ = moderately, 3+ = strongly positive
ND = not done

APPENDIX J

SUPPLEMENTARY DATA

FOR GENOME-WIDE DNA METHYLATION STUDY

(ILLUMINA STUDY)

Table J-1 List of samples for genome-wide DNA methylation study.

Sample number	Type of sample
N082-N	Normal adjacent to CCA, Thailand, matched
U064-N	Normal adjacent to CCA, Thailand, matched
W111-N	Normal adjacent to CCA, Thailand, matched
R052-N	Normal adjacent to CCA, Thailand, matched
W022-N	Normal adjacent to CCA, Thailand, matched
W027-N	Normal adjacent to CCA, Thailand, matched
N082-T	CCA Thailand, matched, short survival
P055-T	CCA Thailand , short survival
P092-T	CCA Thailand , short survival
R103-T	CCA Thailand , short survival
R106-T	CCA Thailand , short survival
T064-T	CCA Thailand , short survival
T098-T	CCA Thailand , short survival
U030-T	CCA Thailand , short survival
U044-T	CCA Thailand , short survival
U059-T	CCA Thailand , short survival
U095-T	CCA Thailand , short survival
W022-T	CCA Thailand, matched, short survival
P060-T	CCA Thailand , long survival
P126-T	CCA Thailand , long survival

Table J-1 List of samples for genome-wide DNA methylation study (cont.).

Sample number	Type of sample
R023-T	CCA Thailand , long survival
R052-T	CCA Thailand, matched, long survival
T005-T	CCA Thailand , long survival
T049-T	CCA Thailand , long survival
U005-T	CCA Thailand , long survival
U021-T	CCA Thailand , long survival
U090-T	CCA Thailand , long survival
W025-T	CCA Thailand , long survival
W111-T	CCA Thailand, matched, long survival
W147-T	CCA Thailand , long survival
R086-T	CCA Thailand , long survival
W027-T	CCA Thailand, matched, long survival
U064-T	CCA Thailand, matched, short survival
0600102A	CCA West
0500031E	CCA West
0900079A	CCA West
0600116A	CCA West
0800213A	CCA West
M055, M139, M213	CCA cell lines, Thailand
MMNK1	Cholangiocyte (normal) cell line, Japan
SKOUA1, HUC	CCA cell lines, West, formalin fixed

Table J-6 Differentially hypomethylated CpG sites out of CpG-islands in immune system process related genes between 6 matched pair samples from Thailand.

Illumina probeID	Chr.	Gene symbol	Average different β value
cg15528736	19	FCGRT||FCGRT	-0.33448
cg04384208	1	FCGR3A||FCGR3A	-0.32899
cg04457794	1	CTSE	-0.30153
cg01963696	19	ELANE	-0.29456
cg02727285	19	RELB	-0.29133
cg26806924	20	PROCR	-0.28971
cg02164442	16	ITGAD	-0.28705
cg11220060	19	KLF1	-0.28054
cg25336198	11	INS||INS-IGF2	-0.26647
cg15928398	3	ST6GAL1	-0.25973
cg13221796	13	RB1	-0.25936
cg17142183	2	IL1R2	-0.25822
cg00839584	2	IL1A	-0.25759
cg01318557	7	LAT2	-0.25691
cg20622019	20	ADA	-0.25551
cg02196805	5	CSF2	-0.25422
cg20530056	1	IKBKE	-0.25255
cg13993218	11	INS||INS-IGF2	-0.25193
cg21478437	1	CTSE	-0.25129
cg19094438	16	BCAR1	-0.25006
cg20394284	9	JAK2	-0.24654

Table J-6 Differentially hypomethylated CpG sites out of CpG-islands in immune system process related genes between 6 matched pair samples from Thailand (cont.).

Illumina probeID	Chr.	Gene symbol	Average different β value
cg13703437	5	FYB	-0.24523
cg12019109	7	AZGP1	-0.24333
cg02149446	19	C5AR1	-0.24081
cg11783497	2	IL1RN	-0.23873
cg15046693	19	CEBPG	-0.23341
cg18854666	2	SLC11A1	-0.23338
cg04425624	6	TNF	-0.23309
cg16777510	17	STAT5A	-0.2324
cg03001305	17	STAT5A	-0.2313
cg23665568	1	BCL10	-0.2306
cg17118262	17	CCL1	-0.22923
cg04567009	1	FCGR3B	-0.22409
cg03375002	7	LAT2	-0.22178
cg10106388	1	CD244	-0.22051
cg10791260	9	TNFSF15	-0.21112
cg26151675	1	CNR2	-0.21051
cg26799474	2	CASP8	-0.20852
cg26589285	19	IL29	-0.20787
cg20340242	2	IL1R2	-0.20665
cg15357945	11	PRG2||PRG2	-0.20178
cg15564267	11	OTUB1	-0.20049
cg06196379	6	TREM1	-0.198

Table J-6 Differentially hypomethylated CpG sites out of CpG-islands in immune system process related genes between 6 matched pair samples from Thailand (cont.).

Illumina probeID	Chr.	Gene symbol	Average different β value
cg21094154	13	TNFSF11	-0.19765
cg00795812	2	PDCD1	-0.19558
cg21432842	17	CSF3	-0.19387
cg02656594	16	IL21R||IL21R	-0.19119
cg00298951	12	CMKLR1	-0.18898
cg18834029	10	RP11-529I10.4||POLL	-0.18853
cg19979896	6	RAET1E	-0.18832
cg07935264	2	IL1B	-0.18727
cg13259290	5	CSF2	-0.18622
cg06214007	1	GBP6	-0.18564
cg16592658	19	EBI3	-0.18121
cg19539004	22	LIF	-0.18111
cg00974864	1	FCGR3B	-0.18093
cg24167037	21	ITGB2	-0.1808
cg23867494	1	TNFRSF4	-0.17472
cg20839149	1	NTRK1||SH2D2A||SH2D2A	-0.17327
cg22424444	11	LOC255512||TOLLIP	-0.17308
cg13500388	16	CBFB	-0.17304
cg10636246	1	AIM2	-0.17289
cg00294382	12	IL23A	-0.17257
cg27606341	5	FYB	-0.16909
cg15077070	2	IL1RL2	-0.16787
cg18431127	15	EPB42	-0.16683

Table J-6 Differentially hypomethylated CpG sites out of CpG-islands in immune system process related genes between 6 matched pair samples from Thailand (cont.).

Illumina probeID	Chr.	Gene symbol	Average different β value
cg01618851	19	CD209	-0.16506
cg07719512	2	SLC11A1	-0.16406
cg15005385	17	CCL3L1||CCL3L3	-0.16075
cg22202141	1	FCGR3A||FCGR3A	-0.1589
cg01980222	6	TREM2	-0.15838
cg10938446	2	IL1RN	-0.15625
cg08244028	5	MSH3||DHFR	-0.15564
cg09179845	5	C7	-0.15418
cg03366382	11	INS||INS-IGF2	-0.15409
cg19486673	19	LILRA2	-0.15106
cg20556988	17	CCL1	-0.14627
cg13742532	3	CCBP2	-0.14433
cg19248557	6	HLA-DRA	-0.13567
cg11809085	9	TNFSF15	-0.13562
cg11484872	6	TNF	-0.13509
cg14580737	19	RFXANK||LOC729991-MEF2B||LOC729991	-0.13421
cg24222324	13	TNFSF11	-0.13371
cg22797169	2	IL1RL2	-0.12594
cg26136776	19	KLF1	-0.12564
cg15952487	1	CD1B	-0.12472

Table J-6 Differentially hypomethylated CpG sites out of CpG-islands in immune system process related genes between 6 matched pair samples from Thailand (cont.).

Illumina probeID	Chr.	Gene symbol	Average different β value
cg11976790	10	DMBT1	-0.12293
cg25623640	4	IGJ	-0.12117
cg11953824	9	C9orf6||IKBKAP	-0.11936
cg06319346	15	CHRFAM7A	-0.11309
cg00626119	1	NTRK1||SH2D2A||SH2D2A	-0.11115
cg20795401	2	ITGA6	-0.10931
cg04790129	21	ITGB2	-0.10589
cg20974196	19	CFD	-0.10135
cg25764570	6	HLA-DRA	-0.09925
cg11207564	17	CCL3L1||CCL3L3	-0.0957
cg26986236	19	GPI	-0.09546
cg09924998	12	VPS33A	-0.09493
cg19921353	6	HLA-DPB1	-0.09417
cg02903525	19	C5AR1	-0.09021

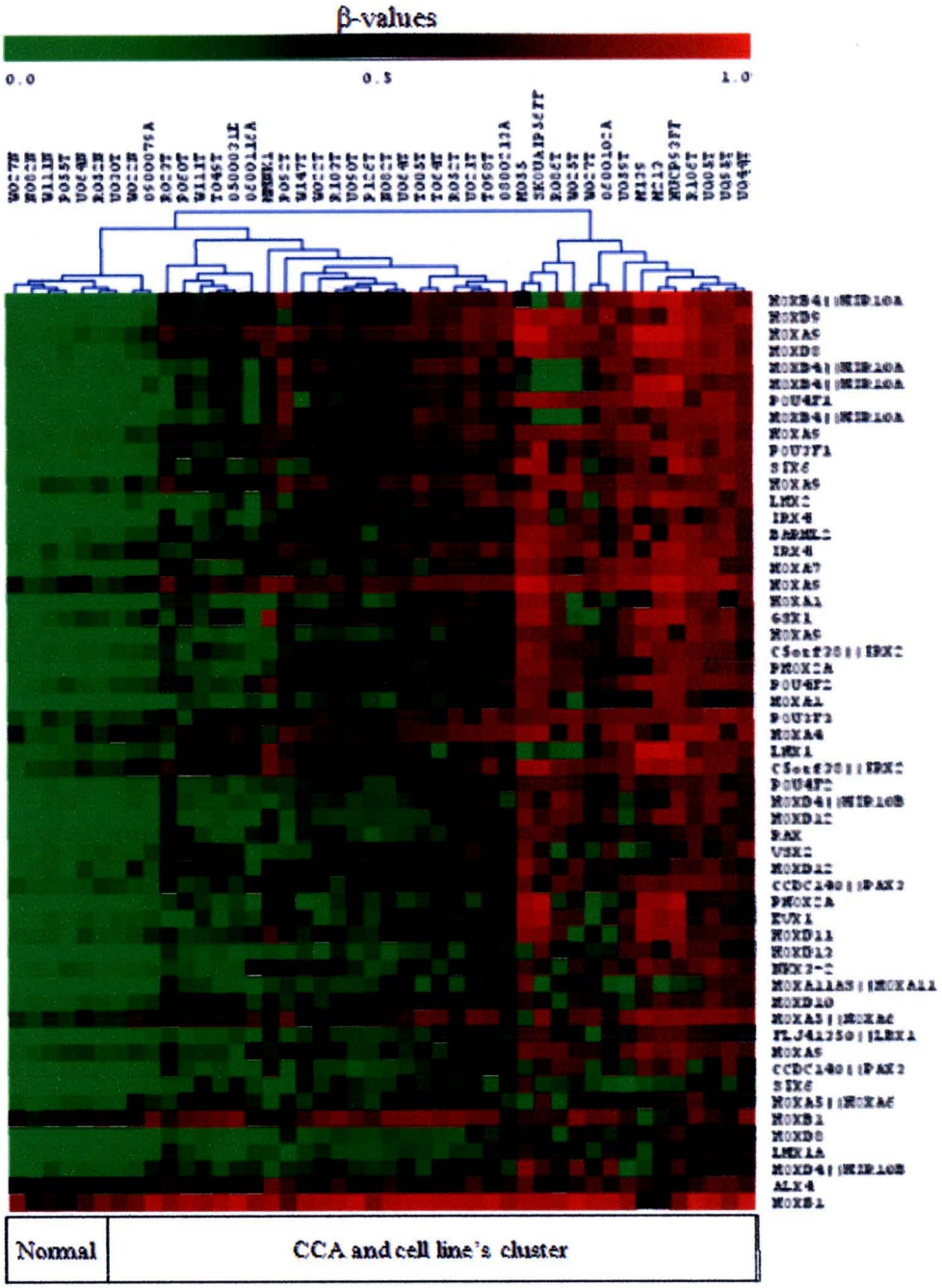
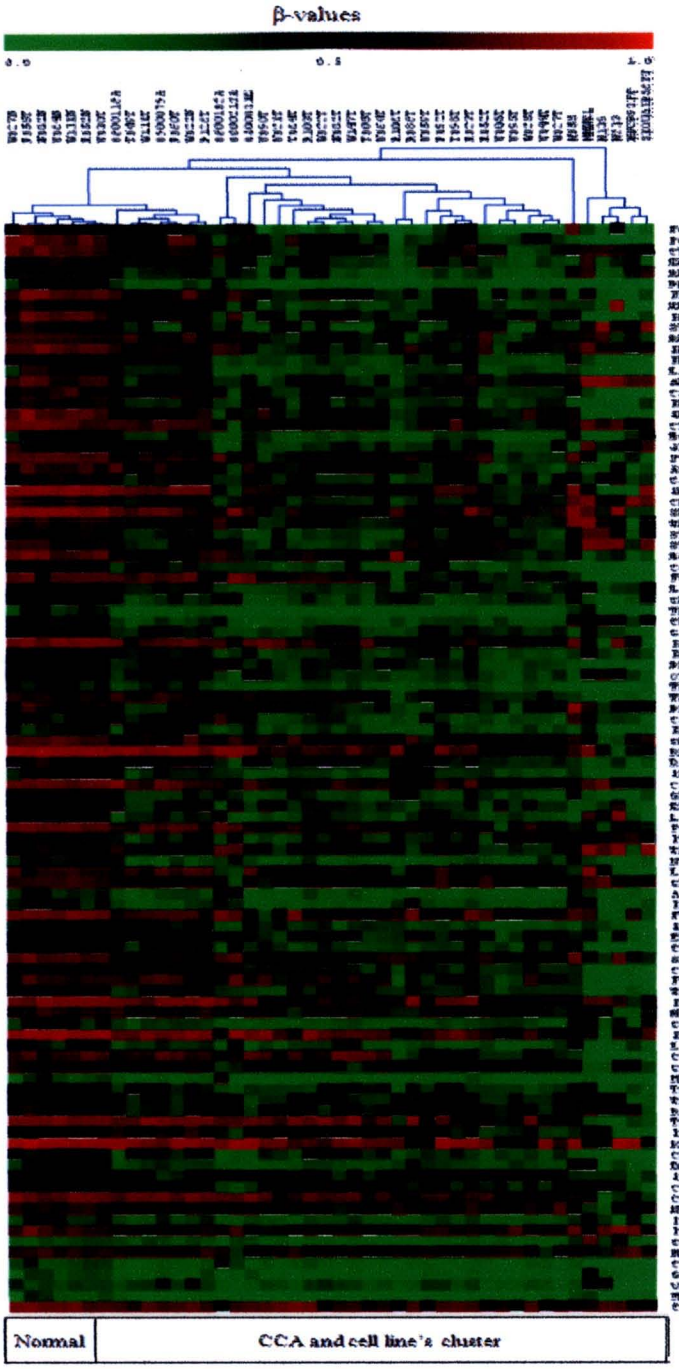


Figure J-1 Hypermethyalted CpG sites within CGI related to *homeobox genes* in the comparison between adjacent normal and CCA (Thailand and West) samples.

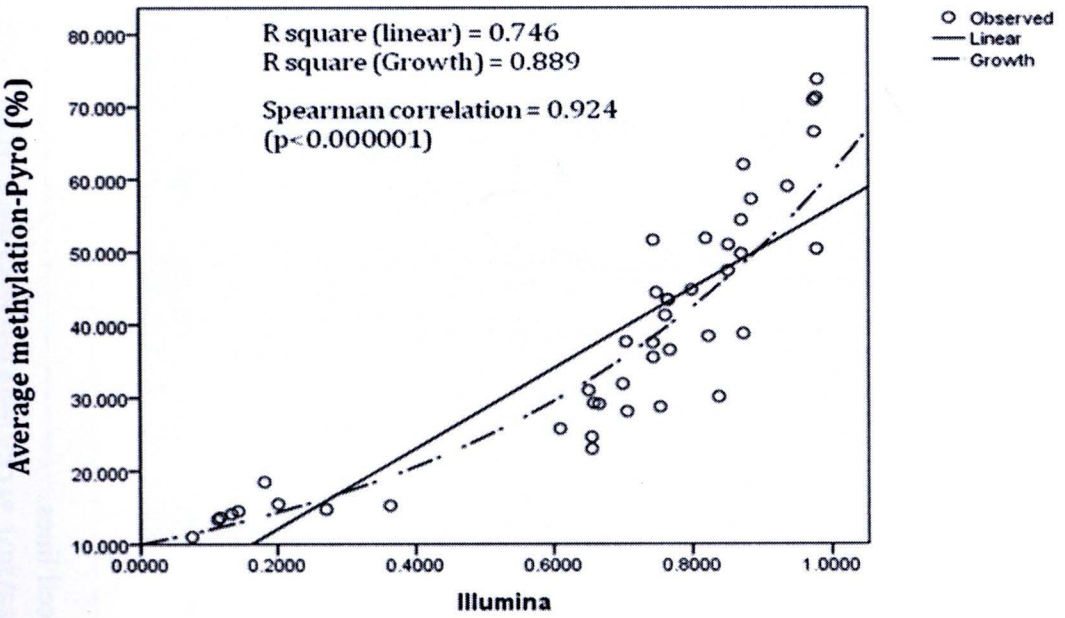


Hypomethyalted CpG sites out of CGI related to immune sy process in the comparison between matched pair samples (adjacent normal vs. CCA, n=6).

APPENDIX K

PYROSEQUENCING RESULTS OF *HOXA9* AND *HOXD9*

A *HOXA9*



B *HOXD9*

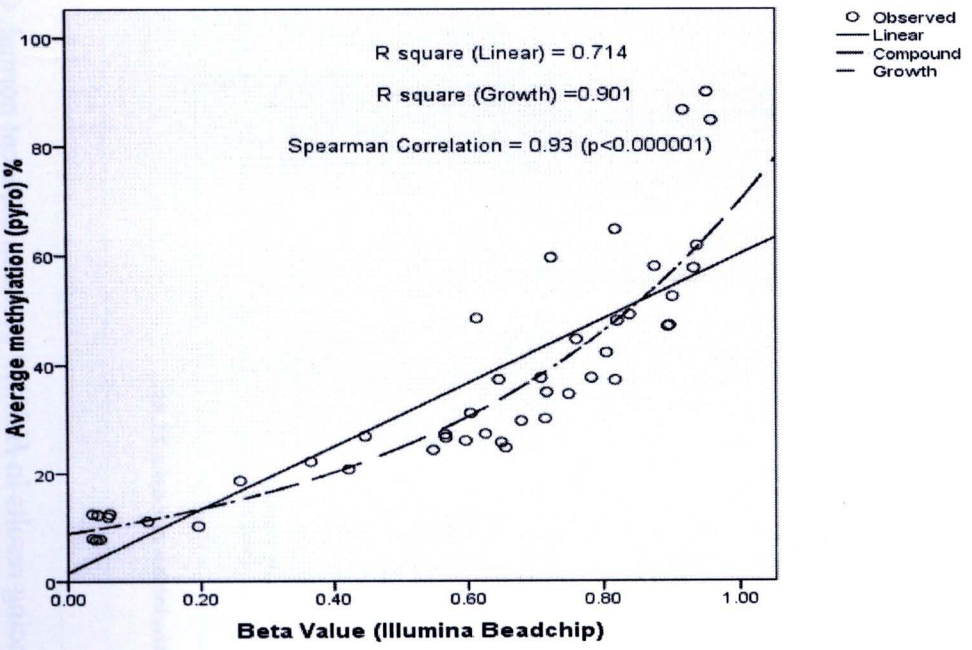


Figure K-1 Correlation of methylation level in *HOXA9* (A) and *HOXD9* (B) between pyrosequencing and Illumina Beadchip assays.

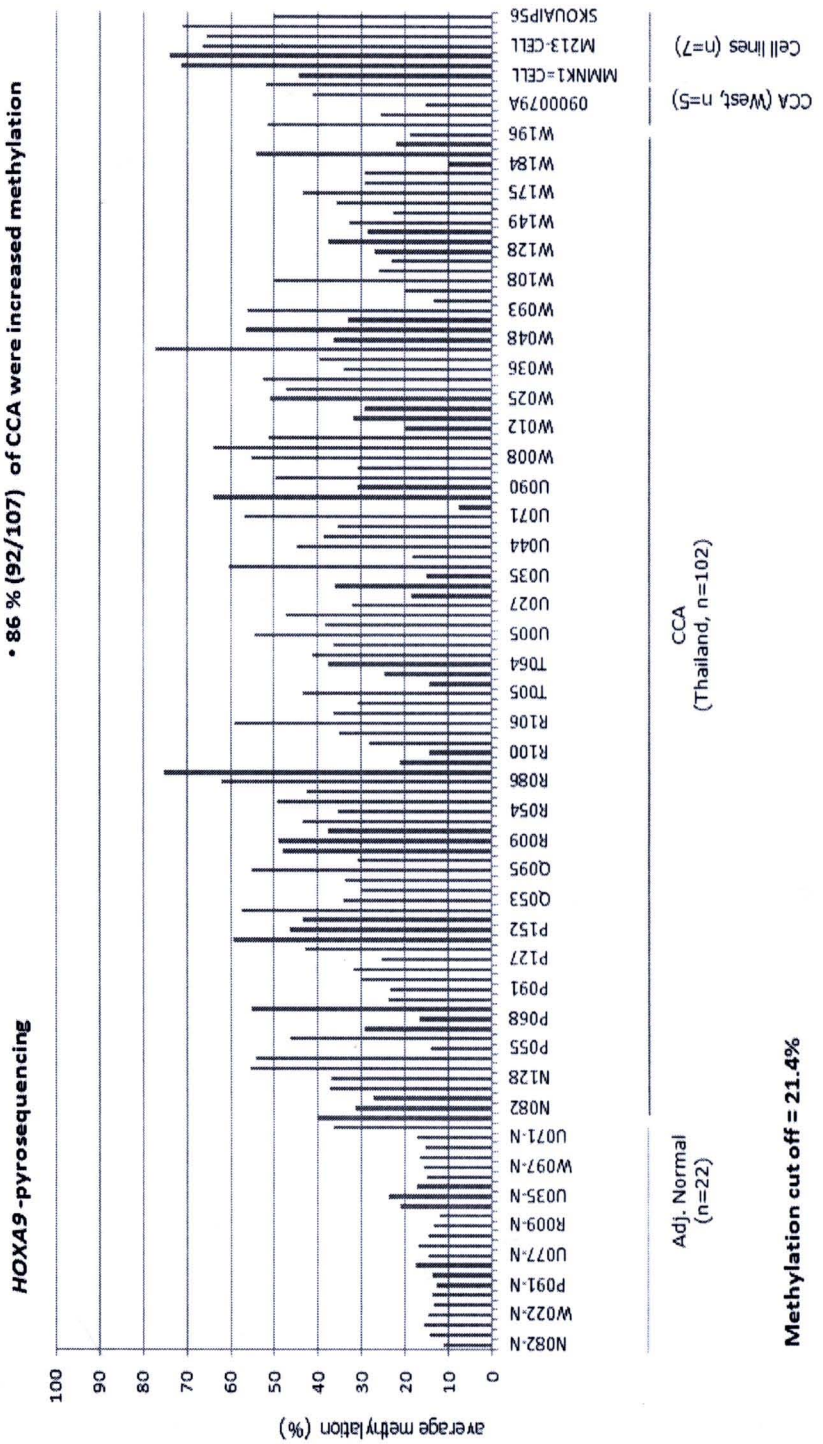


Figure K-2 Pyrosequencing results in *HOXA9* between adjacent normal, CCA and cell lines.

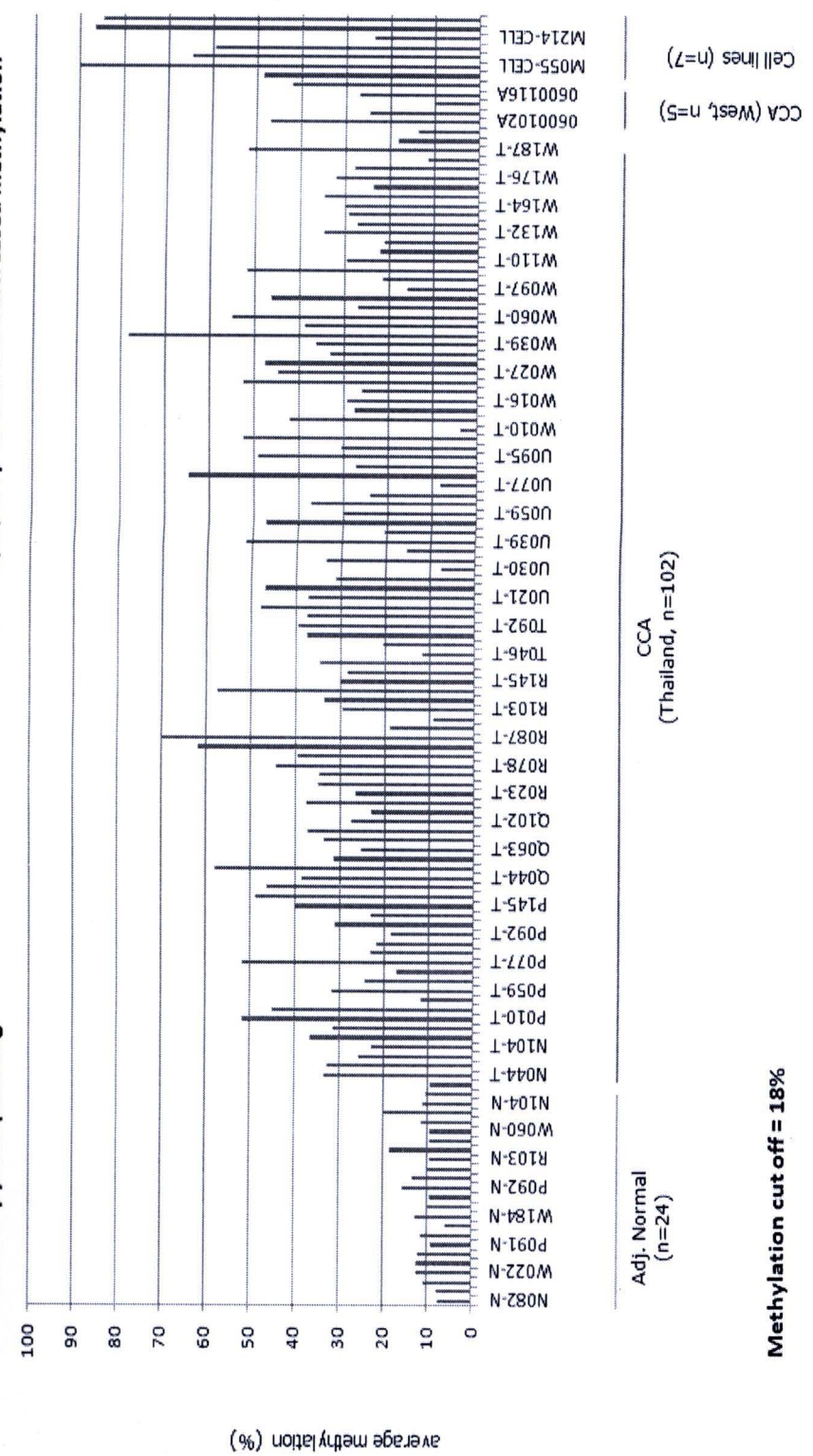


Figure K-3 Pyrosequencing results in *HOXD9* between adjacent normal, CCA and cell lines.

APPENDIX L
RESEARCH PUBLICATION AND PRESENTATION

Research publication and Presentation

1. **Sriraksa R**, Chaopatchayakul P, Jearanaikoon P, Leelayuwat C, Limpaiboon T. Verification of complete bisulfite modification using Calponin-specific primer sets. **Clin Biochem** 2010; 43(4-5): 528-30.

2. **Ruethairat Sriraksa**, Constanze Zeller, Mona A El-Bahrawy, Wei Dai, Jureerut Daduang, Patcharee Jearanaikoon, Vajarabhongsa Bhudisawasdi, Robert Brown, Temduang Limpaiboon. CpG-island methylation profiling in liver fluke-related cholangiocarcinoma. The First Conference of Biomedical Sciences (CBM I): A Novel Concurrence Research. Khon Kaen, Thailand, July 14, 2010 (Oral presentation).

3. Siobhan C. McKay, Kristian Unger, **Ruethairat Sriraksa**, Constanze Zeller, Stephanos Pericleous, Temduang Limpaiboon, Robert Hutchins, Duncan R. Spalding, Bob Brown, Geraldine Thomas. Differing copy number alteration profiles identified by array CGH in Thai Intrahepatic Cholangiocarcinoma related to prognosis. The 61st Annual Meeting of The American Association for The Study of Liver Diseases. Boston, USA, October 29 – November 2, 2010 (co-author).

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Verification of complete bisulfite modification using *Calponin*-specific primer sets

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ABSTRACT

Objectives: To verify complete bisulfite modification.

Design and methods: Bisulfite modified DNA was screened by PCR with *Calponin*-specific primer sets prior to detecting methylation status of *14-3-3σ* by methylation-specific PCR.

Results: False positive methylation was obtained from incomplete bisulfite modification. The lower detection limit of wild type *Calponin*-specific primer is approximately 0.375 ng of unmodified DNA.

Conclusions: Verification of complete bisulfite modification by *Calponin*-specific primer sets can reduce misinterpretation of methylation.

Introduction

Recently, epigenetic alteration has been widely documented as an important mechanism related to carcinogenesis and acquired resistance to therapy. The epigenetic event which affects gene expression by chemical modification includes DNA methylation and histone modification. The hypermethylation of regulatory regions known as CpG islands in some tumor suppressor genes involved in important molecular pathways e.g. cell cycle, apoptosis, DNA repair and cell adhesion, causes their transcriptional inactivation and functional loss [1,2]. Interestingly, the reversibility of DNA methylation provides an opportunity for novel anti-cancer drugs to resensitize epigenetically silenced tumor suppressor genes [3].

Currently, there are numerous methods for detection of DNA methylation such as methylation-specific PCR (MSP), methylation-sensitive single-nucleotide primer extension (Ms-SNuPE), combined bisulfite restriction analysis (COBRA), bisulfite sequencing (BS), MethyLight and methylation microarray [4]. These methods require bisulfite modification which cytosines (C) are converted to uracils (U) while methylated cytosines (5-methylcytosines) remain unchanged [5]. However, if not all cytosines are converted by bisulfite treatment, some of the remaining cytosines might appear as protected by methylation leading to the false positive result [6]. Regarding MSP, PCR band can be detected as virtual methylation occurred in the DNA sequences or false positive due to the remaining unchanged cytosines. Therefore, verification of complete bisulfite conversion is crucial to get virtual methylation status in samples tested. To prove a successful modification of the DNA, a sequence that contains cytosines is amplified after bisulfite modification with primers that will only give an amplified product if the cytosines are successfully converted to uracils. *Calponin* is a constitutive gene highly expressed in smooth muscle and non-muscle cells and found unmethylated in normal condition [7]. In this study, we designed primer sets containing wild type and modified *Calponin*-specific primers which can detect remaining unmodified DNA and complete bisulfite modified DNA, respectively. These primer sets can amplify *Calponin* sequence in the region containing cytosines at non-CpG sites. Using these primer sets, we are able to verify the quality of bisulfite modified DNA in both CpG and non-CpG sites of any allele even those not related to *Calponin*. Therefore, bisulfite modification of *14-3-3σ* could be verified by *Calponin*-specific primers before its methylation status was detected by MSP. The lower detection limit of wild type *Calponin*-specific primer in determining unmodified DNA was also assayed.

Methods

DNA and bisulfite modification

In vitro methyltransferase treated placental DNA was used as a methylated sample. Briefly, 10 μg of human placental DNA was treated with 10 U of SssI methyltransferase (New England Biolabs, Ipswich, MA) containing 160 μM S-adenosylmethionine at 37 °C for 1 h. The sample was incubated at 65 °C for 20 min to stop the reaction,

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CERTIFICATE OF APPRECIATION

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RUETHAIRAT SRIRAKSA

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14 July 2010

Chanvit Leelayuwat.

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Co-Author Disclosure Status

The following authors have completed their AASLD 2010 DDW disclosure:

Siobhan McKay: Disclosure completed

Kristian Unger: Disclosure completed

Ruethairat Sriraksa: No Answer.

Constanze Zeller: No Answer.

Stephanos Pericleous: Disclosure completed

Temduang Limpaiboon: Disclosure completed

Robert Hutchins: Disclosure completed

Duncan Spalding: Disclosure completed

Bob Brown: Disclosure completed

Geraldine Thomas: No Answer.

Abstract Proof

PRESENTATION TYPE: Oral or Poster

CATEGORY: Experimental Hepatobiliary Neoplasia

DESCRIPTORS: F01. Experimental Hepatocarcinogenesis

TITLE: Differing copy number alteration profiles identified by array CGH in Thai intrahepatic Cholangiocarcinoma related to prognosis

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ABSTRACT BODY: Purpose: The worldwide incidence of intrahepatic cholangiocarcinoma has vastly increased over the past three decades, and now represents the second most common primary liver cancer.

Despite this increase in incidence mortality figures for cholangiocarcinoma remain dismal. We employed array comparative genomic hybridization to identify copy number alterations in cholangiocarcinoma with prognostic significance.

Methods: DNA was extracted from 24 Thai cases of intrahepatic cholangiocarcinoma (ICC), and labelled using random primer labelling (Enzo Life Sciences CGH Labeling Kit). Tumour DNA was labelled with Cy3 and sex-mismatched reference DNA (pooled normal DNA, Promega) with Cy5 fluorescence dye and hybridised to 180K Agilent Oligonucleotide array CGH slides for 24 hours. Microarray slides were scanned at 3 micron (Agilent), fluorescence intensities extracted using the Feature Extraction Software (Agilent) and subsequently analysed within the statistical environment R using the CBS algorithm for segmenation of profiles and the CGHcall package for calling of copy number gains and losses.

Results: Analysis of the data revealed three distinct groups of copy number (CN) alterations related to overall survival. Groups were separated into poor (>4 weeks and <13 weeks, n=9), moderate (>14 weeks and <57 weeks, n=7) and good (>58 weeks, n=8) survival. The good survival group demonstrated very few CN alterations. Gains at 1q and 11q, and losses at 1p and 2q were specific to poor survival. Gains at 1p, 7p and 13p/q, and losses at 11p, 12q and 15p were specific for moderate survival. Several CN alterations were demonstrated in the poor and moderate group, and not found in the good group, including gain at 2q, and losses at 3p, 4p/q, 5q, 6q, 8p and 18p/q. The two most charaterisitc alterations were gain at 1q21.1-24.2 in poor suvivors (p value < 0.04 FDR < 0.11) and gain at 8q24.3 in good survivors (p value < 0.001 and FDR= 0.024).

Conclusion: Intrahepatic cholangiocarcinoma remains a clinical challenge, with limited prognostic information available to patients. We have identified differing copy number alteration profiles in Thai intrahepatic cholangiocarcinoma, separating groups into poor, moderate and good survival.

(No Table Selected)

(No Image Selected)

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