CHAPTER III

PART I: CpG-ISLAND METHYLATION PROFILING IN LIVER FLUKE RELATED CHOLANGIOCARCINOMA

1. Introduction

Cholangiocarcinoma (CCA), a malignancy originating from the biliary epithelium or cholangiocyte, has been considered as a rare disease which accounts for 10-25% of primary liver cancers (including hepatocellular carcinoma, CCA and other) worldwide [103]. However, since the incidence and mortality rates for intrahepatic CCA (ICC) have markedly increased worldwide [2-3], there has been growing interest in this cancer type. Of particular importance is that CCA represents a major public health problem in Thailand where CCA is prevalent in the endemic area of liver fluke infection, especially in the Northeast region. Here, CCA accounts for the highest incidence of this cancer worldwide with truncated age-standardized incidence of ages >35 years up to 317.6 per 100,000 population [4]. The prognosis of CCA is generally poor with less than 5% of patients surviving > 5 years and has not significantly improved over the past three decades [167]. This can partly be attributed to the fact that CCA is notoriously difficult to diagnose and often presents at a late clinical stage. Biomarkers which allow earlier detection of CCA could potentially impact on patient survival by allowing earlier treatment. Although surgical resection can be potentially curative, only a few patients are suitable for surgery due to spread of the disease when late presenting and limited effective non-surgical therapies [5-6].

Epidemiological and experimental evidences implicate the carcinogenic liver fluke as a major risk factor of CCA [8-10, 104]. No stronger relation between a human neoplasm and parasitic infection occurs than that between CCA and infection with the liver fluke, *Opisthorchis viverrini* [104]. It is possible that chronic inflammation driven by the liver fluke infection makes epithelial cells of the biliary tract more susceptible to carcinogens. However, the exact molecular events involved in cholangiocarcinogenesis are not well understood. Like other tumor types, CCA is a result of a multistep process in which genetic and epigenetic aberrations of regulatory

genes are accumulated. For instance, genetic alterations of *K-ras* [21, 105] and *p53* [106-109] have frequently been reported in CCA. Moreover, overexpression of IL-6 [110-111], EGFR [112], COX-2 [113], inducible nitric oxide synthase (iNOS) [114] and MDM-2 [115], as well as decreased expression of TP21 [116] and 14-3-3σ [117] have been shown to provide CCA cells with a growth and survival advantage. In addition, alterations in genes with a function in tumor invasion and metastases such as *E-cadherin, α-catenin, β-catenin* [118-119], matrix metalloproteinase (MMP) [120], and VEGF [112] have been described in CCA. Increased microsatellite instability (MSI) of chromosomal loci at 1p36 and 9p21pter in CCA have been shown [121, 78], indicating abnormalities in the function of DNA mismatch repairing genes in this cancer.

Aberrant DNA methylation at CpG-islands which is associated with transcriptional silencing is widely observed in cancer [12]. DNA methylation is a potential rich source that can potentially be used as diagnostic, prognostic, or predictive biomarkers of clinical outcome. Hypermethylation in CCA have been reported in tumor suppressor genes related to cell cycle regulation (*p16, 14-3-3σ, RASSF1A, p73, p14, p15*), apoptosis (*DAPK, TMS1/ASC, SEMA3B*), cell adhesion (*APC, E-cadherin, TIMP3, THBS1*), and DNA repair and carcinogen metabolism (*hMLH1, MGMT* and *GSTP1*) [73-82] as previously mentioned in Chapter II. However, while these previous studies have reported aberrant methylation at several loci in CCA, the evaluation of potential methylation as diagnostic or prognostic biomarkers is still limited.

Although several studies have conducted DNA methylation of many tumor suppressor genes in CCA, only a few genes have been reported for their methylation status in liver fluke related-CCA. As shown in our previous studies, differences in CCA etiology can result in different prevalence of gene abnormality (82, 109). To address whether the frequency of methylation status of genes in liver fluke related-CCA differed from that of CCA with different etiologies, we have explored the methylation status of genes with diverse functions in liver fluke related-CCA. The methylation status was determined by Methylation-specific PCR (MSP) of a defined set of 26 CpG islands in genes which function in cell cycle regulation, cell growth and survival, apoptosis, cell adhesion, DNA repair and carcinogen detoxification in 102

primary CCA and where possible adjacent normal tissues. Those genes included in our present study, if defected, are well-known to be involved in carcinogenesis, pathogenesis and acquired anti-cancer drug resistance in many tumors. MSP provides a relatively simple and sensitive assay that can be used to assess methylation status in clinical samples moreover loci of interest have been confirmed by bisulfite pyrosequencing or COBRA. We identified differential hypermethylation of a number of CpG-islands and reported their associations with clinicopathological parameters.

2. Materials and methods

2.1 CCA and adjacent normal samples

Frozen liver tissues of 102 primary CCA and 29 tumor-adjacent normal samples were used for DNA extraction. The sample size calculation is shown in Appendix A. Moreover, those CCA samples which had paraffin-embedded sections were also used for immunohistochemistry (n=92). The study was reviewed and approved by the Ethics Committee of Khon Kaen University (Reference No. HE510651).

2.2 Clinicopathological data of CCA patients

Clinicopathological data of CCA patients, including age at initial of diagnosis, gender, gross types, histological differentiated types, and the overall survival time (excluding patients who died within 4 weeks after surgery) were also collected (Table B-1 in Appendix B). All of samples and data were kindly supplied by the Liver Fluke and Cholangiocarcinoma Research Center (LFCRC), Khon Kaen University (Khon Kaen, Thailand). Among CCA samples, mean age at initial diagnosis was 55 years (45.2-64.8 years), and 68 males and 34 females were accounted. All samples were intrahepatic CCA and classified into three gross types; mass-forming (MF, *n*=65), periductal infiltrating (PI, *n*=26) and intraductal-growth (ID, *n*=11) type, and two histological differentiated types; well differentiated (*n*=74) and less differentiated adenocarcinoma (included moderately and poorly differentiated, *n*=25). Median overall survival time of CCA patients was 29 weeks.

2.3 DNA extraction from frozen tissues

Genomic DNA was extracted from primary CCA and adjacent normal tissues using DNeasy Tissue Kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. Each CCA tissue was cut into small pieces and up to 25 mg of tissue was placed in a 1.5 ml microcentrifuge tube and 180 µl Buffer ATL (Qiagen) was added. The chopped tissue was incubated with 20 µl proteinase K at 55 °C until the tissue was completely lysed. RNase treatment was an optional step by adding 4 µl of RNase A (100 mg/ml) and incubation for 2 minutes at room temperature. Two hundred microlitres of Buffer AL (Qiagen) was added into the sample and incubation was taken at 70 °C for 10 minutes. Then, 200 µl of absolute ethanol was added into the cell lysate which was mixed thoroughly by vortexing. The cell lysate mixture was pipetted into the DNeasy Mini spin column placed in a 2 ml collection tube and centrifuged at 10,000 x g for 1 minute. The flow-through solution and collection tube were discarded. The DNeasy Mini spin column is placed in a new 2 ml collection tube, and 500 µl Buffer AW1 (Qiagen) was added into the column. After centrifugation at 10,000 x g for 1 minute, the flow-through solution and collection tube were discarded. The DNeasy Mini spin column was placed in a new collection tube and 500 µl Buffer AW2 (Qiagen) was added. After centrifugation at 16,000 x g for 3 minutes to dry the DNeasy membrane, the flow-through solution and collection tube were discarded. The dried DNeasy Mini spin column was placed in a clean 1.5 ml microcentrifuge tube and 200 µl Buffer AE (Qiagen) was added directly onto the DNeasy membrane, left at room temperature for 1 minute. After centrifugation at 10,000 x g for 1 minute, DNA was eluted into the microcentrifuge tube and the column was discarded. DNA solution was stored at -20 °C until used.

2.4 Cell lines, cell culture and DNA extraction

Seven CCA cell lines were kindly provided by the Liver Fluke and Cholangiocarcinoma Research Center, including KKU-M055, KKU-M139, KKU-M156, KKU-M213, KKU-M214, KKU-100 and OCA-17. Five cell lines were cultured in RPMI 1640 (Gibco-BRL, Ontario, Canada) and other two cell lines (KKU-100 and OCA-17) were cultured in HAM-F12 (Gibco-BRL). All cell lines were

supplemented with 10% fetal bovine serum and incubated at 37 °C with humidified atmosphere of 5% CO_2. Cells were harvested and extracted for genomic DNA by using FlexiGene DNA kit following the manufacturer's protocol (Qiagen).

2.5 *In vitro* methyltransferase treated placental DNA

In vitro methyltransferase treated placental DNA was prepared to use as a methylated positive sample. Briefly, 10 μg of human placental DNA was treated with 10 units of SssI methyltransferase (New England Biolabs, Ipswich, MA) containing 160 μM S-adenosylmethionine at 37 °C for 1 hour. The sample was incubated at 65 °C for 20 min to stop the reaction, followed by ethanol precipitation and resuspended in sterile water.

2.6 Bisulfite modification

DNA is modified by sodium bisulfite, all cytosines (C) are converted to uracil (U), while leaving methylated cytosines (5-methylcytosine) unchanged and remain as cytosine [122]. This modified DNA can then be amplified and sequenced, providing detailed information of the methylation status in all CpG sites within the amplified region [91].

DNA (1 μg) in a volume of 50 μl was denatured by freshly prepared NaOH (final concentration, 0.3 M) at 100 °C for 10 minutes. Thirty microlitres of freshly prepared 10 mM hydroquinone (Sigma, St. Louis, MO) and 620 μl 3 M sodium bisulfite pH 5.0 (Sigma) were added and mixed immediately, and samples under mineral oil were incubated at 50 °C for 16 hours. Modified DNA was then purified using the Wizard DNA Purification resin (Promega, Madison, WI) and eluted with 50 μl of warmed sterile deionized water. Sulfonated uracil was converted to uracil by treatment with NaOH (final concentration, 0.3 M) for 5 minutes at room temperature, followed by ethanol precipitation. DNA was resuspended in 20 μl of sterile deionized water, used immediately or stored at -20 °C. Methylated placental DNA, used as a positive control, was modified by sodium bisulfite prior to MSP analysis.

False-positive MSP results can be obtained if the DNA is incompletely converted during the bisulfite modification [123]. 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. In this study, a region of the *Calponin* promoter was amplified with *Calponin* gene-specific primer sets, modified or wild type *Calponin* gene-specific primers in every modified DNA sample, as the following sequences: modified primers; 5′-GGAAGGTAGTTGAGGTTGTG-3′ (forward) and 5′-CCCAA ACTCAAAACTCTAACCTAAC-3′ (reverse) [124], and wild type primers; 5′-GGA AGGCAGCTGAGGTTGTG-3′ (forward) and 5′-CCCAAGCTCAGGGCTCTGGC CTGGC-3′ (reverse) [125]. Sample which is not found a specific band (333 bp) with modified primers and/or given a specific band with wild type primers is considered unmodified or incompletely modified [124, 125]. The bisulfite conversion reaction was repeated for the samples with unmodified or incompletely modified.

2.7 Detection of methylation status using Methylation-specific polymerase chain reaction (MSP)

2.7.1 Primer sequences for MSP

Methylated-specific primers of 22 CpG-islands for MSP were obtained from methprimerDB (http://medgen.ugent.be/methprimerdb/index.php). In addition, the methylated-specific primers for *14-3-3σ, DcR2, DR4* and *DR5* were obtained from previous studies [73, 126-127]. The sequences of methylated-specific primers, annealing temperature (T_A), $MgCl_2$ concentration used and product size are described in Table 3-1.

Table 3-1 Sequences of methylated-specific primers for MSP with their annealing temperature (T_A), $MgCl_2$ concentration and PCR product sizes.

Gene	Forward primer (5'→3')	T_A (°C)	$MgCl_2$ (mM)	Product size (bp)
1. *APAF-1*	F: TTTCGGGTAAAAGGGATAGAATTAGA	63	3	140
	R: TATAACGCCCTTCCCCCGACGACG			
2. *BLU*	F: TTCGTGGGTTATAGTTCGAGAAAGCG	65	3	157
	R: AACGAATTAACCGCGCCTACGC			
3. *BRCA1*	F: GAGTTTCGAGAGACGTTTGG	63	2	176
	R: AATCTCAACGAACTCACGCC			
4. *CASP8*	F: TAGGGGATTCGGACATTGCGA	55	3	321
	R: CGTATATCTACATTCGAAACGA			
5. *DAPK*	F: GGATAGTCGGATCGAGTTAACGTC	59	2	98
	R: CCCTCCCAAACGCCGA			
6. *DcR1*	F: TTACGCGTACGAATTTAGTTAAC	57	2	127
	R: CATCAAACGACCGAAACG			
7. *DcR2*	F: GGGATAAAGCGTTTCGATC	60	3	139
	R: CGACAACAAAACCGCG			

Table 3-1 Sequences of methylated-specific primers for MSP with their annealing temperature (T_A), $MgCl_2$ concentration and PCR product sizes (cont.).

Gene	**Forward primer (5'→3')**	**T_A (°C)**	**$MgCl_2$ (mM)**	**Product size (bp)**
8. *DR4*	F: TTCGAATTTCGGGAGCGTAGC R: GTAATTCAATCCTCCCCGCGA	60	3	92
9. *DR5*	F: GAGGTAGTGAAAGTATAGTCGCGTC R: CCGAAACGTTCTATCCCCG	60	3	200
10. *FancF*	F: TTTTTGCGTTTGTTGGAGAATCGGGTTTTC R: ATACACCTCAAACCGCCGACGAACAAAACG	65	2	153
11. *Fas*	F: GAAAGGGTAGGAGGTCGGTTTTCGAG R: CACTCTTACGCGAAATCAAAAACGAACTCA	63	2	269
12. *GSTp1*	F: AGTTGCGCGGCGATTTC R: GCCCCAATACTAAATCACGACG	59	2	140
13. *HIC1*	F: AGTTGCGCGGCGATTTC R: AACCGAAAACTATCAACCCTCG	59	1	95
14. *MGMT*	F: TTTCGACGTTCGTAGGTTTTCGC R: GCACTCTTCCGAAAACGAAACG	65	3	81

Table 3-1 Sequences of methylated-specific primers for MSP with their annealing temperature (T_A), $MgCl_2$ concentration and PCR product sizes (cont.).

Gene	Forward primer (5'→3')	T_A (°C)	$MgCl_2$ (mM)	Product size (bp)
15. *MINT25*	F: GCGAAAGCGAAAGTCGTTCG	59	3	213
	R: CCCAACGCACATAACGAACC			
16. *OPCML*	F: GCGCGGTGCGGGTTTATTTC	59	3	135
	R: TCCCGATACCGCCTCGAAACGAACG			
17. *p16*	F: TTATTAGAGGGTGGGGCGGATCGC	67	3	150
	R: GACCCCGAACCGCGACCGTAA			
18. *p21*	F: TAGTACGCGAGGTTTCGGGATC	65	3	197
	R: AACTAACGCAACTCAACGCGAC			
19. *p73*	F: GTTCGCGGTGTTTTTCGCG	61	1	316
	R: AATACCTACCCAACGCTACG			
20. *PTEN*	F: TTAGGGTTGGGAACGTCGGAG	63	4	227
	R: CAACAACCAAAAACCTAACAACGACGACAA			
21. *RASSF1A*	F: CGAGAGCGCGTTTAGTTTCGTT	65	2	192
	R: CGATTAAACCCGTACTTCGCTAA			

Table 3-1 Sequences of methylated-specific primers for MSP with their annealing temperature (T_A), $MgCl_2$ concentration and PCR product sizes (cont.).

Gene	Forward primer (5'→3')	T_A (°C)	$MgCl_2$ (mM)	Product size (bp)
22. *SFRP-1*	F: CGTATTTTAGTTTTGTAGTTTTCGG	61	4	163
	R: CCCCCGACCAATAACG			
23. *SOCS-3*	F: TTTTGTGGATTTTACGGTCGT	59	4	134
	R: GAAAAACTAATCCCGAATCGAA			
24. *Survivin*	F: TCTGTATATTTCGCGTCGTTTC	61	2	280
	R: AAACCGAACAATCTCACCCGCT			
25. *TMS1*	F: TTGTAGCGGGGTGAGCGGC	65	1	191
	R: AACGTCCATAAACAACAACGCG			
26. *14-3-3 σ*	F: TGGTAGTTTTTATGAAAGGCGTC	59	2	105
	R: CCTCTAACCGCCCACCACG			

2.7.2 Methylation specific PCR

MSP was performed on a PTC-100 Thermal cycle (MJ Research INC., Ramsey, MN, USA). Bisulfite-modified DNA was used as a template for PCR. A total volume of 25 μL of PCR contained 1X PCR buffer (16.6 mM ammonium sulfate and 67 mM Tris, pH 8.8), appropriate concentration of $MgCl_2$ for each primer set, 10 mM 2-mercaptoethanol, 200 μM each of dNTPs, 5 pmole of each primer, and 150 ng of bisulfite-treated DNA. The reactions were hot-started at 95 °C for 5 minutes and 1.5 units of *Taq* polymerase was added during this initial phase. Amplification was performed for 35 cycles; denaturation at 95 °C for 30 seconds, appropriate annealing temperature of each primer set for 30 seconds, and extension at 72 °C for 30 seconds. The final extension was incubated at 72 °C for 10 minutes. For MSP, bisulfite modified DNA of *in vitro* methyltransferase treated placental and normal leukocyte DNA were used as methylated positive and unmethylated control, respectively. Sterile water was added instead of DNA template as negative control for PCR. Ten microlitres of PCR products were separated by 2% agarose gel electrophoresis and visualised under UV illuminator after ethidium bromide staining. Methylation positive sample was defined by comparison of MSP band of sample to that of diluted placental DNA treated *in vitro* with methyltransferase (positive control) and very low intensity signal was disregarded.

2.8 Pyrosequencing

Pyrosequencing of bisulfite modified DNA was used to validate methylation status of candidate loci, *OPCML and DcR1*, previously identified by MSP. The primers for *OPCML* amplification and sequencing were as follows; 5′-GGGAGTGTGAGATGTATGTGAGTG-3′ (forward), 5′-biotin-TACCCCCAAAAC CACAACTAATT-3′ (reverse) and 5'-AGAGGTAGGTTTGTTGTGT-3' (sequencing primer), and for *DcR1*; 5′-TTGGTAGTGTAGTTGTGGGAATTTTT-3′ (forward), 5′-biotin-TCTATCCCCAAAATTCCCTAA-3′ (reverse) and 5'-GGTAGTGTAGTT GTGGGAA-3' (sequencing primer). The schematics of these specific primers for pyrosequencing are presented in Figures F-1A and B (Appendix F). DNA template was bisulfite-converted using EpiTect® Bisulfite Kit (Qiagen, West Sussex, UK). The

annealing temperature and $MgCl_2$ concentration for amplification of *OPCML and DcR1* were 63 °C, 2 mM and 61 °C, 3.6 mM, respectively. We used the PyroGold Reagent kit (Biotage, Uppsala, Sweden) on a Pyrosequencing 96HS plate according to the manufacturer's instruction. In brief, 10 µL PCR products for each sequencing reaction were immobilized with streptavidin-coated beads (Streptavidin Sepharose HP, GE Healthcare, Amersham, UK) in Binding Buffer (Biotage) for 5 minutes. The biotinylated template was purified with the Pyrosequencing vacuum prep tool (Biotage) and incubated with 10 pmol per reaction individually with each sequencing primer in Annealing Buffer (Biotage) on sequencing plate. The sequencing plate was incubated at 80°C for 2 minutes to denature DNA strands and re-annealed with sequencing primer at room temperature. Sequencing was performed following the manufacturer's protocol. A Pyrogram was generated and analyzed by the Pyro Q-CpG™ software (Biotage). In the Pyrogram, methylcytosine (^{m}C) and cytosine (C) are represented as C and T peaks, respectively, which give quantitative information about the methylation percentage at an individual CpG site.

2.9 Combined bisulfite restriction analysis (COBRA)

COBRA was performed to validate methylation stutas of *OPCML*, *SFRP1* and *HIC1*. Briefly, bisulfite modified DNA was used as a template for PCR with specific primers for each loci. A total volume of 25 µL PCR reaction was denatured at 95 °C for 5 minutes. Then, amplification was performed for 45 cycles; denaturation at 95 °C for 30 seconds, appropriate annealing temperature of each primer set for 30 seconds, and extension at 72 °C for 30 seconds. The final extension was incubated at 72 °C for 5 minutes. Primer sequences and PCR condition for each CpG-island are shown in Tables 3-2 and 3-3, respectively.

Table 3-2 Primer sequences of PCR for COBRA of *OPCML*, *SFRP1* and *HIC1*.

CpG-island	Primer sequences (5'→3')
OPCML	F: GTTTTTTTTGTAGGGGAAGT
	R: CAACAACTCCATCCCTAACC
SFRP1	F: CGTATTTTAGTTTTGTAGTTTTCGG
	R: CCCCCGCCAATAACG
HIC1	F: TAGTTGGAAAATTTTTTTTTAAGTTTG
	R: AATTACCCCAATTAAAAAAAATAATAC

Table 3-3 PCR conditions for COBRA of *OPCML*, *SFRP1* and *HIC1*.

Reagents	CpG-island		
	OPCML	*SFRP1*	*HIC1*
Sterile H_2O (μl)	17.8	17.8	17.8
10X PCR buffer (μl)	2.5	2.5	2.5
25 mM $MgCl_2$ (μl)	2	2	2
150 ng/μl *SFRP1* primer mix (μl)	1	1	1
10 mM dNTPs (μl)	0.5	0.5	0.5
Fast start *Taq* DNA polymerase (μl)	0.2	0.2	0.2
Bisulfite modified DNA (μl)	1	1	1
Total	25 μl/reaction		
Annealing temperature (°C)	59	53	53
Product size (bp)	243	163	443

After amplification, PCR products were purified using the QIAquick PCR purification kit (Qiagen) according to the manufacturer's instructions. The eluted products were digested with restriction enzyme which cuts at the retained site of methylated alleles. PCR products of *OPCML*, *SFRP1* and *HIC1* were digested with Fnu4HI$^{(M)}$, BsiEI$^{(M)}$ and Hpy118III$^{(M)}$, respectively. The components of one digestion reaction are shown in Table 3-4.

Table 3-4 The conditions for digestion with restriction enzyme for COBRA analysis.

Reagents	Volume (μl)
Eluted PCR product	14μl
10x NEB buffer 4	2μl
Restriction enzyme	1μl
100X BSA	0.2μl
Sterile H_2O	To 20μl

Digestion was performed on a heating block at 37°C for 2 hours. Following digestion, products were separated on a 2% agarose gel (150 volts, 90 minutes) and band intensity was evaluated for level of methylation using software of ONE-Dscan one dimension gel analysis (version 2.05, Scanalytics, Fairfax, VA). In each CpG-island, COBRA was performed on 15 CCA samples, 4 CCA cell lines, and controls (100% *in vitro* methylated DNA IVM, 75% IVM, 50% IVM and 25% IVM, and normal leukocyte DNA N) to validate methylation status of each CpG-island compared to MSP results.

2.10 Protein expression analysis by immunohistochemistry

The protein expression of OPCML and DcR1 was studied by immunohistochemistry on formalin fixed paraffin-embedded tissue sections of 92 primary CCA samples. The sections were dewaxed in xylene and rehydrated through descending concentrations of ethanol series and then water. Then, endogenous peroxidase was blocked using 0.6% hydrogen peroxide solution for 15 minutes. Antigen retrieval was performed by microwaving tissue sections in EDTA (pH 9.0) for 20 minutes. To block non-specific antibody binding, sections were incubated with 2.5% normal horse serum (Vector Laboratories, Peterborough, UK) for 10 minutes. Sections were incubated overnight at 4 °C with OPCML monoclonal mouse antibody (1:1000 dilution; Anti-human OBCAM: MAB27771, R &D Systems) or DcR1 monoclonal mouse antibody (1:100 dilution; DcR1 (L19): sc-73890, Santa Cruz Biotechnology). A section of each sample was incubated with PBS instead of primary

antibody as negative control and an appropriate positive control (breast carcinoma) was also tested every round of staining. After incubation, sections were washed with PBS containing 0.1% Tween-20, followed by incubation for 30 minutes at room temperature with secondary antibody (ImmPRESS Universal Antibody (anti-mouse Ig/anti-rabbit Ig, peroxidase) Polymer Detection Kit, Vector Laboratories) and then washed with PBS. Thereafter, diaminobenzidine chromogen was applied to visualize the reaction and sections were next counterstained with haematoxylin. All slides were examined and scored by two independent observers. The staining was scored as negative (=0), or positive with weak (=1), moderate (=2), and strong (=3) intensity.

2.11 Statistic analysis

The statistic analysis was performed using SPSS software (SPSS version 13.0, Chicago, IL, USA). The correlation between methylation of each CpG-island and cliniclopathological features of CCA patients including gender, gross types, and histological differentiated types was analysed using chi-square test or Fisher's exact test. The correlation between methylation status and age at initial of diagnosis was analysed using Mann-Whitney U test. The association of methylation status and overall survival time was determined using Kaplan-Meier method by log-rank test or univariate analysis by Cox regression. The association between MSP and pyrosequencing, COBRA or protein expression was analysed using chi-square test. In addition, we defined a methylation index (MI) as the ratio between the number of methylated CpG-islands and the number of analysed CpG-islands [128]. The concurrent methylation at the loci of interest was analysed by comparing the methylation status of that gene (methylated or unmethylated) with the MI calculated from remaining CpG-islands and also determined the association with clinicopathological data. $P < 0.05$ was considered as statistically significant.

3. Results

3.1 Frequency of CpG-island methylation in cholangiocarcinoma and adjacent normal tissues

CpG-island hypermethylation of 26 loci was investigated in 102 primary CCA and 29 matched adjacent normal tissues using MSP. Hierarchical clustering showed that highly methylated loci were clustered together the same as the clusters of intermediate, low and unmethylated loci, respectively (Figure 3-1) and the histogram of methylation frequency in gene profile was shown as Figure 3-2. All bisulfite treated-DNA samples were tested with *Calponin*-specific primer sets to demonstrate successful bisulfite modification prior to MSP analysis [124-125]. CpG-islands with high frequency of methylation in CCA included *14-3-3σ*, *OPCML*, *SFRP-1*, *HIC1*, *PTEN,* and *DcR1* (81.4, 72.5, 63.7, 38.2, 35.3, and 28.4%, respectively). Moderately frequent methylation was found in *MINT25*, *p16*, *RASSF1A,* and *BLU* (15.7, 15.7, 14.7, and 10.8%, respectively). *DAPK*, *CASP8*, *FAS*, *MGMT*, and *p73* were methylated in 1-10% of CCA while no CpG-island methylation was found in *APAF-1*, *BRCA1*, *DcR2*, *DR4*, *DR5*, *FancF*, *GSTP1*, *p21*, *SOCS-3*, *Survivin,* and *TMS1*. Adjacent normal tissues showed no or slightly low percentage of methylation, with the exception of *14-3-3σ* in which high frequency of methylation (75.9%) was found and excluded from further data analysis. Excluding *14-3-3σ*, 91% (93/102) of CCA samples were methylated in at least one locus out of 14 loci (*OPCML*, *SFRP-1*, *HIC1*, *PTEN*, *DcR1*, *MINT25*, *p16*, *RASSF1A*, *BLU*, *DAPK*, *CASP8*, *FAS*, *MGMT* and *p73*). The statistic analysis of difference between methylation frequency in CCA and adjacent normal samples was described in Table E-1 (Appendix E).

Furthermore, we also determined methylation status of 7 CCA cell lines (KKU-M055, KKU-M139, KKU-M156, KKU-M213, KKU-M214, KKU-100 and OCA-17) at 26 CpG islands. As shown in Table 3-5, highly frequent methylation was found in *OPCML, HIC1, SFRP-1, MINT25, p16, DcR1* and *PTEN* in CCA cell lines (100, 100, 85.7, 85.7, 85.7, 71.4 and 57.1% of tested cell lines, respectively). These finding indicated that frequencies of those highly methylated CpG-islands were similar in CCA cell lines and primary tumors.

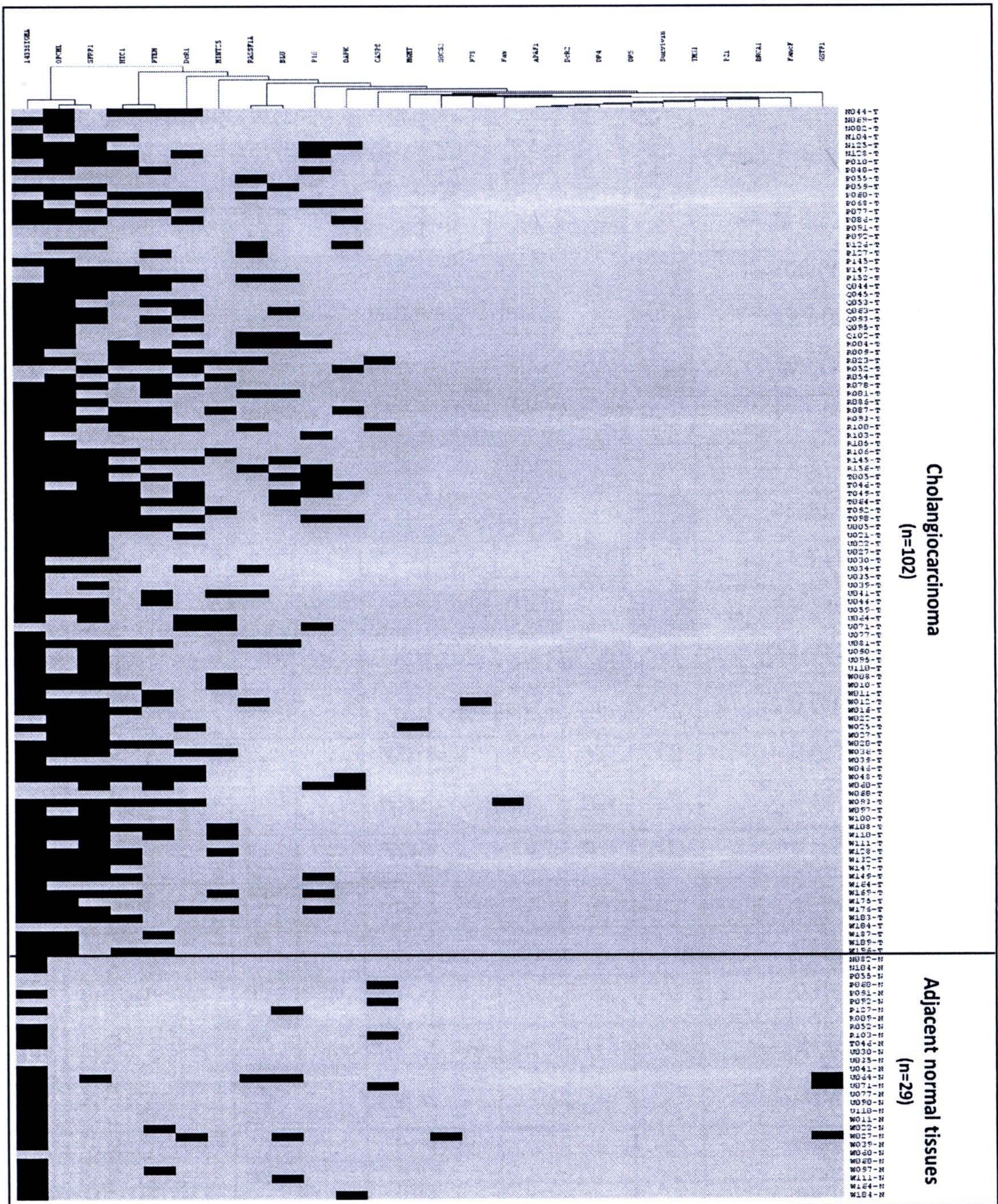

Figure 3-1 Methylation profile of 26 CpG-islands in cholangiocarcinoma and adjacent normal samples. Black and grey boxes represent positive and negative methylation by MSP. Twenty-six CpG-islands included *14-3-3σ, OPCML, SFRP1, HIC1, PTEN, DcR1, MINT25, RASSF1A, BLU, P16, DAPK, CASP8, MGMT, SOCS3, P73, Fas, APAF1, DcR2, DR4, DR5, Survivin, TMS1, p21, BRCA1, FancF, GSTP1*, respectively.

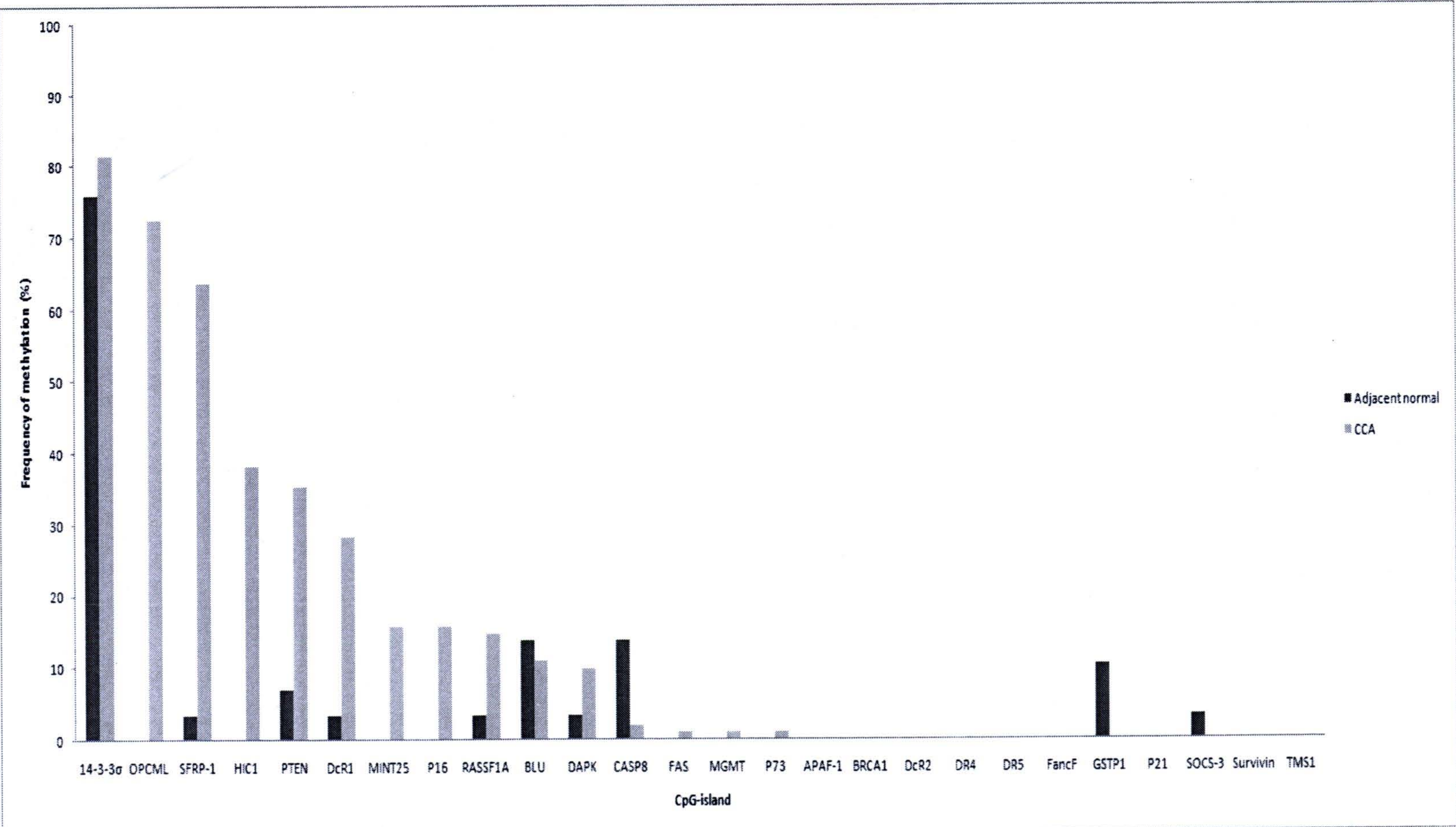

Figure 3-2 Frequency of methylation of 26 CpG-islands in adjacent normal samples (n=29) and cholangiocarcinoma (n=102).

Table 3-5 Methylation of selected methylated CpG-islands in CCA cell lines.

CCA cell line / CpG-island	M055	M139	M156	M213	M214	KKU-100	OCA-17	% methylation (n_{meth} / n_{tested})
14-3-3σ	+	-	-	-	-	+	-	28.6 (2/7)
OPCML	+	+	+	+	+	+	+	100 (7/7)
SFRP-1	+	+	+	+	+	-	+	85.7 (6/7)
HIC1	+	+	+	+	+	+	+	100 (7/7)
PTEN	-	-	+	+	+	+	-	57.1 (4/7)
DcR1	+	+	+	-	+	+	-	71.4 (5/7)
MINT25	+	w+	+	+	+	-	w+	85.7 (6/7)
RASSF1A	-	-	-	-	-	+	-	14.3 (1/7)
p16	+	w+	+	+	w+		w+	85.7 (6/7)
BLU	-	-	-	-	-	w+	-	14.3 (1/7)
DAPK	-	-	-	-	-	-	-	0 (0/7)
DcR2	-	-	**ND**	-	-	**ND**	**ND**	0 (0/4)
DR4	-	-	**ND**	-	-	**ND**	**ND**	0 (0/4)
DR5	-	-	**ND**	-	-	**ND**	**ND**	0 (0/4)
CASP8	-	-	-	-	-	-	-	0 (0/7)
FAS	-	-	-	-	-	-	-	0 (0/7)
MGMT	-	-	-	-	-	-	-	0 (0/7)
P73	-	-	-	-	-	-	-	0 (0/7)
APAF1	-	-	-	-	-	-	-	0 (0/7)
BRCA1	-	-	-	-	-	-	-	0 (0/7)
FancF	-	-	-	-	-	-	-	0 (0/7)
GSTP1	-	-	-	-	-	-	-	0 (0/7)
P21	-	-	-	-	-	-	-	0 (0/7)
SOCS3	-	-	-	-	-	-	-	0 (0/7)
Survivin	-	-	-	-	-	-	-	0 (0/7)
TMS1	-	-	-	-	-	-	-	0 (0/7)

+, positive methylation; +w, weakly positive methylation; -, negative; ND, not done

3.2 Concurrent methylation of muliple CpG-islands in CCA

Concurrent methylation has been reported as potentially indicative of a methylator phenotype or selection for common epigenetic events during tumor development [129-130].

The number of loci methylated in tumors but not in normal adjacent tissue ranged from one to seven. Of 102 cases of CCA, 64 cases (62.8%) were methylated in three or more CpG-islands. Furthermore, 45 cases (44.1 %) of CCA were found methylation of four or more CpG-islands whereas only 6.9% of adjacent normal tissue had methylation of four or more loci (Figure 3-3A). Moreover, the average methylation index of CCA, corresponding to three CpG-islands of 25 analysed loci, was significantly higher than that in adjacent normal tissues (average MI; 0.12 vs. 0.02, $P < 0.001$) as shown in Figure 3-3B.

Concordant methylation in CCA was found between *OPCML* and *SFRP-1* (P=0.007), *OPCML* and *HIC1* (<0.001), and *HIC1* and *PTEN* (P=0.002) (Figure 3-4). No significant association between other pairs of CpG-island was observed. The difference of methylation index between methylated and unmethylated status of 5 highly methylated CpG-islands (*OPCML*, *SFRP1*, *HIC1*, *PTEN* and *DcR1*) was also determined in CCA. The significant difference of MI was found in CCA with methylated *OPCML*, *HIC1*, *PTEN* or *DcR1* but not *SFRP1* compared to unmethylated (Figure 3-5).

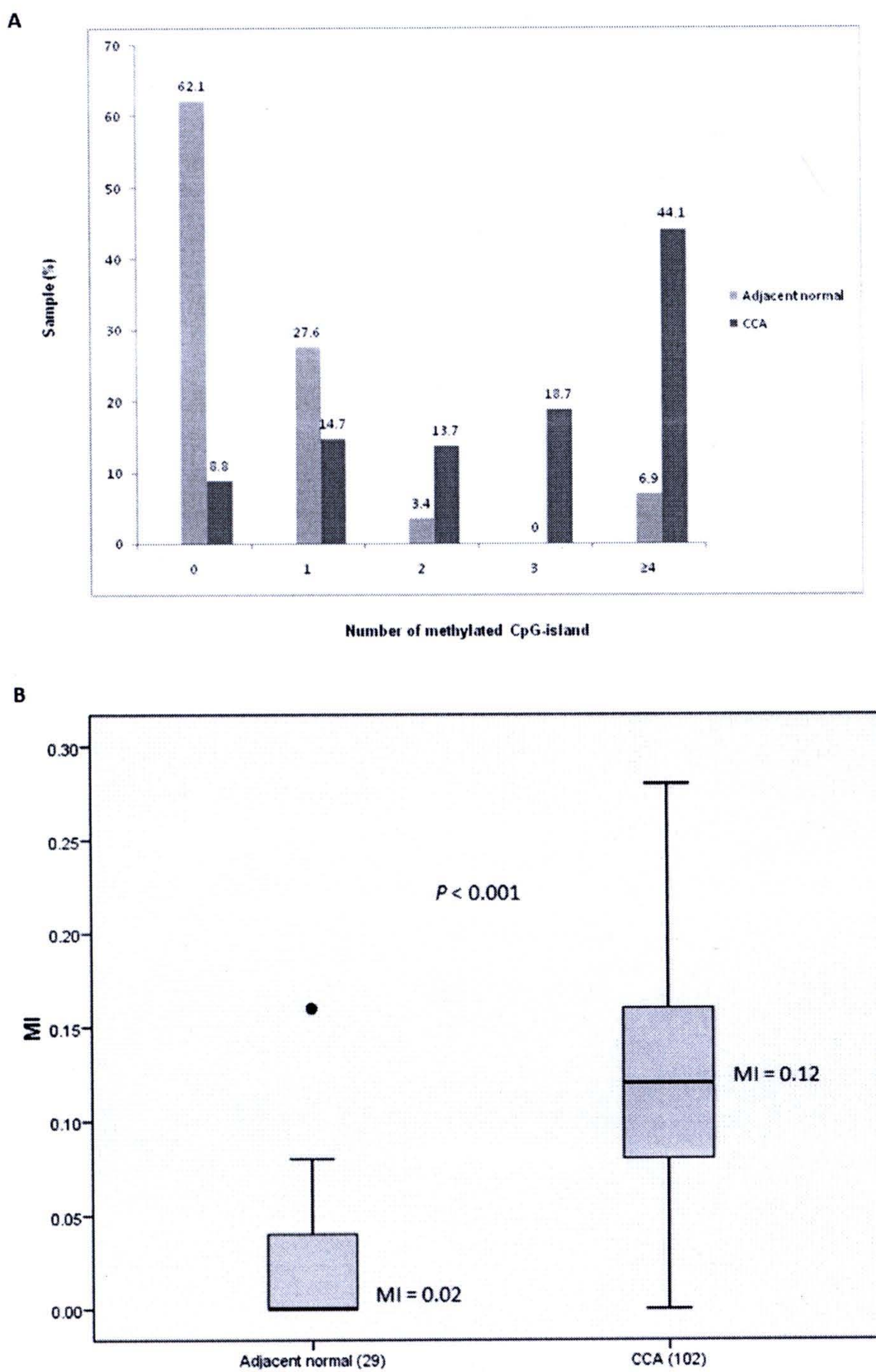

Figure 3-3 Number of methylated CpG-island (A) and methylation index; MI (B) in adjacent normal samples and cholangiocarcinoma.

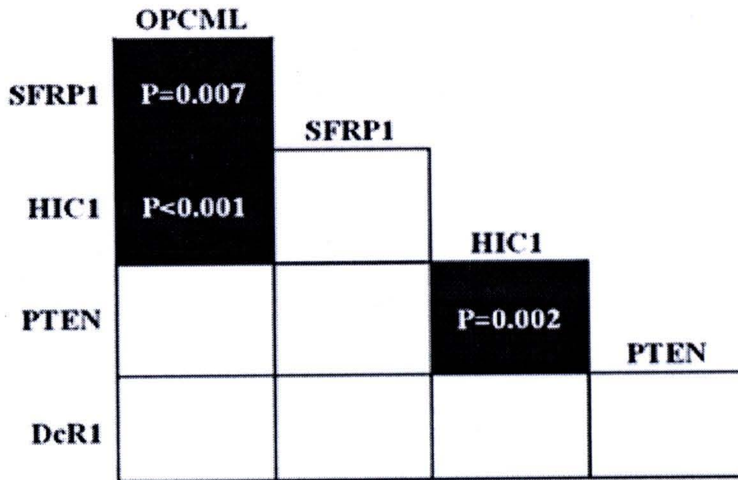

Figure 3-4 Concurrent methylation of highly methylated CpG-islands in cholangiocarcinoma. The pair of CpG-islands with $P < 0.01$ represents the significant association of methylaiton in each pair analysed by bivariate association (Pearson's correlation coefficients).

A

P< 0.001
MI = 0.12
MI = 0.06
MI
Unmethylated OPCML (28) Methylated OPCML (74)
OPCML

B

P = 0.175
MI = 0.11
MI = 0.09
MI
Unmethylated SFRP1 (37) Methylated SFRP1 (65)
SFRP1

C

P< 0.001
MI = 0.15
MI = 0.09
MI
Unmethylated HIC1 (63) Methylated HIC1 (39)
HIC1

D

P= 0.012
MI = 0.14
MI = 0.10
MI
Unmethylated PTEN (66) Methylated PTEN (36)
PTEN

E

P< 0.001
MI = 0.16
MI = 0.10
MI
Unmethylated DcR1 (73) Methylated DcR1 (29)
DcR1

Figure 3-5 Methylation index (MI) between methylated and unmethylated status of 5 highly methylated CpG-islands. CCA samples with methylation of *OPCML*, *HIC1*, *PTEN,* or *DcR1* had significantly higher MI than CCA with unmethylation of those loci (A, C-E), but no significant difference in *SFRP1* (B).

3.3 Correlation between CpG-island methylation and clinicopathological data

Correlation between CpG-island methylation and clinicopathological data of CCA patients including age at initial of diagnosis, gender, gross type, histological type and overall survival time, was examine. The result showed that CCA patients with methylated *PTEN* were older than those without (mean; 57.8 vs. 53.3 years, *P*=0.027). Only *DcR1* was more frequently methylated in female than male (female; 41.2% (14/34) and male; 22.1% (15/68), *P*=0.044). Methylation of *MINT25* was associated with gross type and found more frequently in intraductal-growth type than other types (ID; 45.5% (5/11), PI; 15.4% (4/26), MF; 10.8% (7/65), *P*=0.014). According to differentiated types of CCA, methylation of *OPCML* was found more frequently in less differentiated type (less differentiation; 88% (22/25) and well differentiation; 67.6% (50/74), *P*=0.047). As shown in Figure 3-6, CCA patients with methylation of *DcR1* had longer overall survival than those without (Median; 41.7 vs. 21.7 weeks, *P*=0.027). However, all of these associations are of only borderline significance, multiple testing should be taken into account and further validation in larger independent studies is required. Given the potential association of *DcR1* with survival, we also determined methylation status of CpG islands at other TRAIL related genes (*DcR2*, *DR4* and *DR5*) but did not observe methylation in any sample tested. The detailed association between methylation status of gene profile and clinicopathological data is shown in Table 3-6.

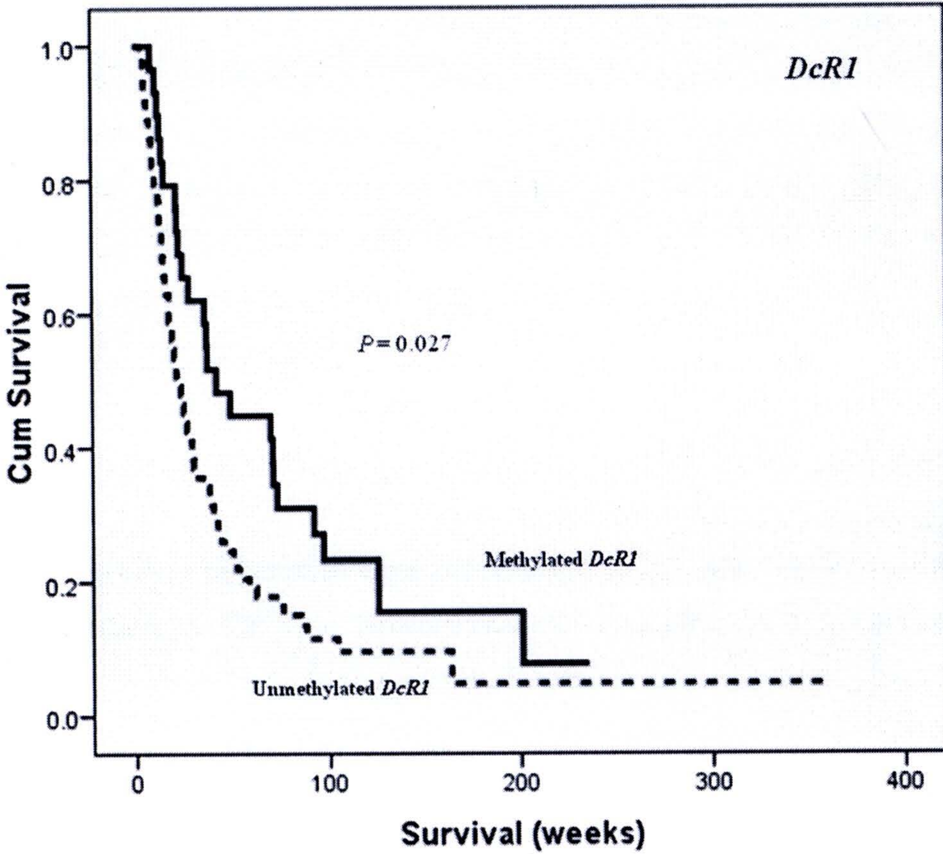

Figure 3-6 The association between methylation of *DcR1* and overall survival time of cholangiocarcinoma patients. Patients with methylated *DcR1* had longer overall survival time than those without (Kaplan-Meier analysis using log-rank test, P=0.027).

Table 3-6 Association between methylation of gene profiling and clinocopathological data.

Gene	**%Methylation (n_{pos}/n_{tested})**	**Clinicopathological data**				
		Age[1]	**Gender[2]**	**Gross[2]**	**Histological[2]**	**Survival[3]**
14-3-3σ	81.4 (83/102)	NS	NS	NS	NS	NS
OPCML	72.5 (74/102)	NS	NS	NS	$P = 0.047$	NS
SFRP-1	63.7 (65/102)	NS	NS	NS	NS	NS
HIC1	38.2 (39/102)	NS	NS	NS	NS	NS
PTEN	35.3 (36/102)	$P = 0.027$	NS	NS	NS	NS
DcR1	28.4 (29/102)	NS	$P = 0.044$	NS	NS	$P = 0.027$
MINT25	15.7 (16/102)	NS	NS	$P = 0.014$	NS	NS
P16	15.7 (16/102)	NS	NS	NS	NS	NS
RASSF1A	14.7 (15/102)	NS	NS	NS	NS	NS
BLU	10.8 (11/102)	NS	NS	NS	NS	NS
DAPK	9.8 (10/102)	NS	NS	NS	NS	NS
CASP8	1.9 (2/102)	NS	NS	NS	NS	NS
FAS	0.98 (1/102)	NS	NS	NS	NS	NS
MGMT	0.98 (1/102)	NS	NS	NS	NS	NS
P73	0.98 (1/102)	NS	NS	NS	NS	NS
APAF-1	0 (0/102)	NS	NS	NS	NS	NS
BRCA1	0 (0/102)	NS	NS	NS	NS	NS
DcR2	0 (0/102)	NS	NS	NS	NS	NS
DR4	0 (0/102)	NS	NS	NS	NS	NS
DR5	0 (0/102)	NS	NS	NS	NS	NS
FancF	0 (0/102)	NS	NS	NS	NS	NS
GSTP1	0 (0/102)	NS	NS	NS	NS	NS
P21	0 (0/102)	NS	NS	NS	NS	NS
SOCS-3	0 (0/102)	NS	NS	NS	NS	NS
Survivin	0 (0/102)	NS	NS	NS	NS	NS
TMS1	0 (0/102)	NS	NS	NS	NS	NS

NS = no statistical significance, [1] T-test, [2] Chi-square test, [3] Kaplan-Meier test

3.4 Validation of methylation status of candidate CpG-islands

Pyrosequencing and COBRA were used to confirm methylation status of *OPCML* and *DcR1* observed in CCA by MSP. For pyrosequencing data, we also determined the cut-off of methylation level (each CpG site and average) from adjacent normal samples using non-parametric method to defined methylation status into negative or positive methylation (Table F-2, Appendix F). The correlation between MSP and pyrosequencing or COBRA results was analysed using Chi-square test and significant association with MSP was found at *OPCML* by COBRA (80.0% of tested samples, P=0.025), Table 3-7. However, primers for pyrosequencing could not be optimised for use at the same site as the MSP primers. For *DcR1*, MSP methylation status was significantly validated by bisulfite pyrosequencing, both at each CpG site and average of methylation (4 CpG sites) as shown in Table 3-8. There was a trend for methylation of CpG1 to be correlated with survival of CCA patients (Cox-regression; P=0.08) (Table F-1, Appendix F), similar to that previously seen by MSP.

Table 3-7 Validation of methylation status of *OPCML* using pyrosequencing and COBRA compared to MSP.

***OPCML*-Pyrosequencing**	**% validated result compared to MSP ($n_{validated}/n_{tested}$)**	***P****
CpG1	60.8 (62/102)	0.405
CpG2	53.9 (55/102)	0.452
CpG3	57.8 (59/102)	0.219
CpG4	62.8 (64/102)	0.784
CpG5	66.7 (68/102)	0.711
CpG6	53.9 (55/102)	0.452
Average	62.8 (64/102)	0.784
***OPCML*-COBRA**	80.0 (12/15)	0.025

* Chi-square test

Table 3-8 Validation of methylation status of *DcR1* using pyrosequencing compared to MSP.

***DcR1*-Pyrosequencing**	**% validated result compared to MSP ($n_{validated}/n_{tested}$)**	***P****
CpG1	79.6 (78/98)	<0.001
CpG2	77.6 (76/98)	0.001
CpG3	87.8 (86/98)	<0.001
CpG4	87.8 (86/98)	<0.001
Average	82.7 (81/98)	<0.001

* Chi-square test

3.5 Expression of OPCML and DcR1

To address whether methylation has effect on gene expression, immunohistochemisrty was performed to determine OPCML and DcR1 protein expression in CCA samples. As expected, less or no OPCML and DcR1 protein expression was detected in CCA, in which 63% (58/92) showed weakly expressed OPCML and 26% (24/92) had no expression (Table 3-9), the same as DcR1, which 54% (50/92) showed low DcR1 expression and 23% (22/92) exhibited no expression (Table 3-10). Low protein expression was found in 88% (59/67) of *OPCML*-hypermethylated CCA and 77% (20/26) of *DcR1*-hypermethylated samples. The significant correlation was not found between methylation and protein expression from both genes. Since the vast majority of tumors had no or very low OPCML and DcR1 expression, it was not feasible to examine for association between expression and methylation. Interestingly, expression of DcR1 was significantly associated with gross types (P=0.01), which 100% (11/11) of ID type had low DcR1 expression, followed by MF and PI types with 82.5% (47/57) and 58.3% (14/24), respectively.

Table 3-9 Protein expression and methylation status of *OPCML*.

IHC / MSP	Low expression		High expression		Total
	Neg	**1+**	**2+**	**3+**	
Unmethylation	7	16	2	0	25
Methylation	17	42	8	0	67
Total	24	58	10	0	92

Chi-square test; *P*=0.86

Table 3-10 Protein expression and methylation status of *DcR1*.

IHC / MSP	Low expression		High expression		Total
	Neg	**1+**	**2+**	**3+**	
Unmethylation	34	18	7	7	66
Methylation	16	4	5	1	26
Total	50	22	12	8	92

Chi-square test; *P*=0.322

4. Discussion

In the present study, the majority of CCA (91%) shows aberrant methylation in at least one locus. Moreover, CCA has significantly higher methylation index than adjacent normal biliary samples and the concurrent methylation of multiple loci is also observed among highly methylated loci in the tumors, suggesting that DNA methylation is a common event in CCA which agreed to previous reports [73-74]. However, CCA samples of those previous studies are not liver fluke related tumors as the set of samples used in our recent study. The high methylation frequency of *14-3-3σ* found in CCA and adjacent normal tissues, similarly to that in normal and breast cancer [131] suggests that methylation of *14-3-3σ* is tissue specific regardless of pathological status. The methylation status of selected loci detected by MSP was correlated with pyrosequencing or COBRA, indicating the reliability of MSP. The high methylation frequency found in *OPCML*, *SFPR1*, *HIC1*, *PTEN* and *DcR1* suggests that many signalling pathways are involved in the development and progression of CCA. Moreover, association of methylated *OPCML* and *DcR1* with patient clinicopathological data indicates their role in tumor progression, which hypermethylation results in low or no protein expression. *OPCML* (Opioid binding protein/cell adhesion molecule-like gene) belongs to the IgLON family of immunoglobulin (Ig) domain containing glycosylphosphatidylinositol (GPI)-anchored cell adhesion molecules that are involved in cell adhesion and cell-cell recognition [132]. In addition, *OPCML* has been proposed as a stress- and p53-responsive gene, with the response impaired when promoter becomes hypermethylated. Moreover, ectopic expression of OPCML in carcinoma cells lacking its expression leads to dramatic growth inhibition suggesting *OPCML* as a tumor suppressor [133]. *OPCML* hypermethylation has been reported in several cancers e.g. 33.3% of late stage ovarian cancer [124], 70% of hepatocellular carcinoma [134], 63.9% of invasive cervical cancer [135] and 57-100% of multiple carcinomas and lymphomas [133]. We are the first to report *OPCML* methylation in CCA with high frequency (72.5%) but no methylation was found in normal. The previous studies have demonstrated that CCA with less differentiation shows a poorer outcome and high incidence of metastases than well differentiation [21, 136]. Taken together, loss of OPCML reduces the intercellular adhesion and thus promotes tumor progression. The high incidence of

OPCML methylation in CCA and its association with less differentiation together with its absence in normal implicate that *OPCML* methylation could be used as an epigenetic biomarker for molecular prognosis [21, 136] and diagnosis of CCA. Further study of *OPCML* methylation in biliary diseases should be done to validate its use as a diagnostic marker specifically to CCA.

The highly frequent methylation of *SFRP-1*, *HIC1* and *PTEN* found in our CCA cases suggested that epigenetic silencing of these tumor suppressor genes may lead to an increase in cell proliferation and survival. The secreted frizzled-related protein 1 (SFRP1) is a soluble Wnt antagonist which contains a cysteine-rich domain homologous to Frizzled receptor [137-138]. This enables SFRP1 to downregulate Wnt/ β-catenin signalling by competing with Frizzled for Wnt binding or by binding directly to Frizzled [139]. *SFRP1* is inactivated frequently by promoter hypermethylation in many cancers including CCA [82], esophageal adenocarcinoma [140], breast cancer [141] and hepatocellular carcinoma [142]. Ectopic expression of SFRPs downregulates T-cell factor/lymphocyte enhancer factor (TCF/LEF) transcriptional activity in liver cells, while overexpression of β-catenin mutant and depletion of SFRP1 using siRNA synergistically upregulates TCF/LEF transcriptional activity suggesting that functional loss of SFRPs by hypermethylation contributes to activation of Wnt signalling during hepatocarcinogenesis [143]. It is therefore conceivable that abrogation of *SFRP1* by hypermethylation is important in cholangiocarcinogenesis.

The hypermethylated in cancer 1 (*HIC1*) is located on the short arm of chromosome 17 (17p13.3) telomeric to the *p53* tumor suppressor gene [144]. It encodes a transcriptional suppressor targeting on histone deacetylase *SIRT1*, proneural transcriptional factor *ATOH1*, cell cycle and apoptosis regulator *E2F1*, G-protein-coupled receptor *CXCR7*, and cell surface ligand *ephrin-A1*. Interestingly, deregulation of these target genes in the lack of HIC1 function is related to widespread effects in both development and cancer growth [145-149]. The HIC1-SIRT1-p53 circular regulatory loop has been proposed in which HIC1 directly represses the transcription of *SIRT1* deacetylase resulting in increased p53 acetylation. Active acetylated p53 binds to the p53-responsive elements and trans-activates its downstream target genes including *HIC1* [145, 150]. Frequent hypermethylation of

HIC1 has been reported in various human cancers e.g. breast cancer [151], colorectal cancer [152], hepatocellular carcinoma [153] and ovarian cancer [154]. Down-regulation and hypermethylation of *HIC1* are associated with tumor aggressiveness and poor overall survival in many human cancers [155-158]. It is conceivable that mutational inactivation (41.6%) and LOH of p53 (32%) in liver fluke related-CCA as shown in our previous studies together with p53 deacetylation due to *HIC1* methylation (38.2%) impede p53 function which allows cells to bypass apoptosis and survive DNA damage [108-109]. Phosphatase and tensin homologue deleted on chromosome 10 (PTEN) is a phosphatase specific for the 3-position of the inositol ring. Its primary target is phophatidylinositol-3, 4, 5 triphosphate (PIP3), the direct product of phosphatidylinositide 3-kinase (PI3K) [159-160]. Loss of PTEN function either by mutations, deletions or promoter hypermethylation can cause the accumulation of PIP3 which triggers the activation of PI3K and its downstream effectors, PDK1, Akt/PKB and mTOR resulting in cell cycle progression, survival, metabolism and migration through the phosphorylation of many physiological substrates [161-162]. By contrast to the study of Lee *et al* (2002), which no PTEN methylation was found in Korean CCA samples, we demonstrated high PTEN methylation (35.3%) in our CCA cases suggesting that inactivation of PTEN by hypermethylation is a major cause of molecular carcinogenesis and pathogenesis of liver fluke related CCA, confirming our previous studies that differences in genetic and epigenetic alteration patterns are dependent on aetiologies of CCA [78, 121].

Although the defect of programmed cell death through p53-dependent pathway has been observed in CCA, it is surprising that death receptor-induced apoptosis was rarely methylated. These findings indicate the advantage for selective cancer treatment and good outcome in CCA patients. The decoy receptors have been postulated to account for TRAIL resistance as overexpression of *DcR1* and/or *DcR2* prevent cancer cells from TRAIL-induced apoptosis. Hypermethylation of *DcR1* (28.4%) but not death receptors (*DR4* and *DR5*) and other TRAIL signalling related genes (*APAF-1*, *CASP8*, and *DcR2*) in our study indicates the use of recombinant TRAIL or TRAIL receptor agonistic monoclonal antibodies as selective anti-tumor therapy. To overcome TRAIL resistance that always happened, combined treatment with standard chemotherapeutics can enhance TRAIL-induced apoptosis. Previous

studies showed correlation of methylated *DcR1* with improved prognosis in malignant mesothelioma and prostate cancer [163-164]. As longer overall survival was observed in methylated *DcR1* patients, *DcR1* methylation may be useful as a prognostic marker of CCA. No methylation at any locus tested was found in 9 CCA samples. This may be due to less tumor cells presented in these samples since 4 samples showed < 10% of tumor cells.

In conclusion, promoter hypermethylation has been observed in many genes which play important roles in carcinogenesis and progression of liver fluke related CCA. Further study is warranted to validate the use of high frequency methylated genes as potential biomarkers for diagnosis, prognosis and prediction of CCA patients. Moreover, tumor cells are significantly more sensitive to TRAIL-induced apoptosis than normal cells, thus the use of recombinant TRAIL or TRAIL receptor agonistic monoclonal antibodies for selective treatment of CCA in combination with chemotherapeutic drugs may improve patient survival.