

ห้องสมุดงานวิจัย สำนักงานคณะกรรมการวิจัยแห่งชาติ



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**CpG-ISLAND METHYLATION PROFILING IN
CHOLANGIOCARCINOMA**

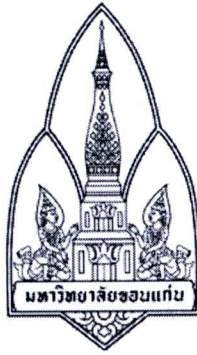
MISS RUETHAIRAT SRIRAKSA

**A THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY
KHON KAEN UNIVERSITY**

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MISS RUETHAIRAT SRIRAKSA

**A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF
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GRADUATE SCHOOL KHON KAEN UNIVERSITY**

2010



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ฤทัยรัตน์ ศรีรักษา. 2553. **ภาวะเมทิลเลชันบริเวณ CpG-island ของชุดจีนในมะเร็งท่อน้ำดี.**

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ถึงแม้ว่าความผิดปกติทางพันธุกรรมในระดับ genetic ที่เกี่ยวข้องกับมะเร็งท่อน้ำดีจะมีรายงานกันอย่างกว้างขวาง แต่อย่างไรก็ตามความผิดปกติในระดับ epigenetic ยังคงมีการศึกษาอย่างจำกัด ดังนั้นเราจึงได้ทำการศึกษาภาวะเมทิลเลชันบริเวณ CpG-island ของชุดจีน จำนวน 26 CpG-islands ในโรคมะเร็งท่อน้ำดีที่เกี่ยวข้องกับการติดเชื้อพยาธิใบไม้ในตับ โดยตรวจหาภาวะเมทิลเลชันในตัวอย่างมะเร็งท่อน้ำดี จำนวน 102 ตัวอย่าง และเนื้อเยื่อท่อน้ำดีปกติในบริเวณใกล้เคียง จำนวน 29 ตัวอย่าง โดยวิธี methylation specific PCR (MSP) หลังจากนั้นทำการตรวจยืนยันภาวะเมทิลเลชันของจีนที่พบว่ามีความสัมพันธ์อย่างมีนัยสำคัญกับข้อมูลทางพยาธิวิทยาคลินิกของผู้ป่วยมะเร็งท่อน้ำดีโดยใช้วิธี pyrosequencing และ/หรือ combined bisulfite restriction analysis (COBRA) รวมทั้งตรวจหาการแสดงออกของจีนดังกล่าวโดยวิธี immunohistochemistry จากการศึกษาค้นคว้าครั้งนี้พบว่าจีนที่เกิดภาวะเมทิลเลชันมากกว่า 25% ของตัวอย่างมะเร็งท่อน้ำดี ได้แก่ OPCML, SFRP1, HIC1, PTEN และ DcR1 ซึ่งมีความแตกต่างอย่างมีนัยสำคัญเมื่อเทียบกับเนื้อเยื่อท่อน้ำดีปกติ นอกจากนี้พบว่า 91% ของตัวอย่างมะเร็งท่อน้ำดีมีภาวะเมทิลเลชันของจีนใดจีนหนึ่งในจำนวน 25 จีนที่ทำการศึกษา (ยกเว้น 14-3-3c ซึ่งไม่พบความแตกต่างของเมทิลเลชันในมะเร็งและเนื้อเยื่อท่อน้ำดีปกติ จึงไม่ใช้ในการวิเคราะห์ข้อมูล) และพบว่าตัวอย่างมะเร็งท่อน้ำดีมีการเกิดเมทิลเลชันร่วมกันของหลายจีนมากกว่าในเนื้อเยื่อท่อน้ำดีปกติอย่างมีนัยสำคัญทางสถิติ (Methylation index (MI); 0.12 และ 0.02, $P < 0.001$) เมื่อเปรียบเทียบการเกิดเมทิลเลชันร่วมกันของหลายจีนระหว่างตัวอย่างมะเร็งท่อน้ำดีที่มีเมทิลเลชันและไม่มีเมทิลเลชันในจีนที่พบภาวะเมทิลเลชันสูง พบว่า ตัวอย่างมะเร็งท่อน้ำดีที่มีเมทิลเลชันของจีนใดจีนหนึ่ง ได้แก่ OPCML, HIC1, PTEN หรือ DcR1 มีโอกาสเกิดเมทิลเลชันร่วมกันของจีนอื่นๆ ที่ทำการทดสอบอีกหลายจีน (MI; 0.12-0.16) ซึ่งมากกว่าผู้ป่วยมะเร็งที่ไม่มีเมทิลเลชันของจีนใดจีนหนึ่งใน 4 จีนดังกล่าวอย่างมีนัยสำคัญ ในการศึกษาครั้งนี้พบว่า จีนที่เกิดเมทิลเลชันสูงที่สุด คือ OPCML ซึ่งพบใน 72.5% ของตัวอย่างมะเร็งท่อน้ำดีแต่ไม่พบในเนื้อเยื่อท่อน้ำดีปกติ และมักเกิดเมทิลเลชันใน less differentiated CCA สูงกว่า well

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Asst. Prof. Dr. Jureerut Daduang

ABSTRACT

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Although genetic changes have been associated with cholangiocarcinogenesis, epigenetics changes are poorly characterised. Firstly, we aimed to elucidate CpG-island hypermethylation in liver fluke related-CCA which may have potential as diagnostic or prognostic biomarkers. Methylation status of 26 CpG islands associated with candidate genes associated with tumorigenesis were analysed in 102 primary CCA and 29 matched adjacent normal biliary samples using methylation specific PCR (MSP). Then, loci of interest were further characterised by pyrosequencing and/or combined bisulfite restriction analysis (COBRA), as well as protein expression by immunohistochemistry. We have identified a number of CpG-islands (OPCML, SFRP1, HIC1, PTEN and DcR1) with >25% frequency of differential hypermethylation in CCA. The results showed that 91% of CCA were methylated in at least one CpG-island. By comparison, average methylation index (MI) of CCA was significantly higher than that in adjacent normal samples (MI; 0.12 vs. 0.02, $P < 0.001$). In addition, concurrent methylation was found among highly methylated loci, with methylation of OPCML, HIC1, PTEN or DcR1 having significantly higher number of other loci methylated (MI; 0.12-0.16, corresponding to ≥ 3 CpG-islands) than patients without methylation of one out of those four CpG-islands. The most frequently methylated locus (OPCML) was methylated in 72.5% of tumors and is more frequently methylated in less differentiated CCA compared to well differentiated tumors. Patients with methylated DcR1 had significant longer overall survival (Median; 41.7 vs. 21.7 weeks, $P = 0.027$). Low protein expression was found in 88% of OPCML-hypermethylated CCA and 77% of DcR1-hypermethylated samples, respectively. Thus, aberrant hypermethylation is a common event in liver fluke related-CCA and may potentially contribute to aberration of many genes involved to molecular pathways. OPCML might serve as a suggestive diagnostic

methylation marker and its association to less differentiated tumors and DcR1 is associated with good outcome in CCA patients.

To extend the analysis of methylation of candidate loci, we investigated genome-wide DNA methylation signature of CCA. Genome-wide DNA methylation study in CCA was performed using HumanMethylation27 BeadChip on 32 primary CCA, 6 matched adjacent normal samples, 5 CCA cell lines and a normal biliary cell line. Differentially hypermethylated and hypomethylated CpG sites located within and out of CpG-islands were identified. DNA methylation profiles were visualized using hierarchical clustering. Specific functional term and gene set enrichment analyses (GSEA) were performed to identify the utility and significant function of genes associated with the CpG sites showing differential methylation. Moreover, we validated the reliability of Methylation BeadChip data by comparing the methylation status of selected genes obtained from pyrosequencing. We have reported the genome-wide DNA methylation profiles in CCA showing the changes both hypermethylation and hypomethylation of hundreds of CpG sites within and out of CpG-islands. A number of specific functional terms in the genes associated with differentially methylated CpG sites were identified including homeobox genes. Moreover, a number of gene sets were significantly associated with hypermethylation in CCA including homeobox genes, EED target, PRC2_targets1, SUZ12 targets and H3K27 targets, whereas OCT4 targets, PRC2 and NOS targets have been significantly hypomethylated in tumors. The epigenetic aberrations of these genes have proposed to play a role in stem cell origin of cancers, probably, including CCA. The reliability of methylation data from the BeadChip has been found with strong correlation comparing to pyrosequencing on selected homeobox gene, HOXA9 and HOXD9 ($P < 0.001$), with $>85\%$ of primary CCA were increased methylation of both loci compared with adjacent normal samples. DNA methylation signatures with hypermethylation and hypomethylation in multiple CpG sites have been found being changed as a common molecular aberration in CCA. To our knowledge, this is the first report of genome-wide DNA methylation study in cholangiocarcinoma which provides a useful resource of epigenetic signature which could serve as the biomarkers. However, the biological functions and clinical application of these candidate DNA methylation changes should be further clarified.

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**This thesis is Dedicated to My Parents, Brothers,
Entire Teaching Staff, Friends, and Cancer patients**

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Ruethairat Sriraksa

TABLE OF CONTENTS

	Page
ABSTRACT (IN THAI)	i
ABSTRACT (IN ENGLISH)	iii
DEDICATION	v
ACKNOWLEDGEMENTS	vi
TABLE OF CONTENTS	vii
LIST OF TABLES	xi
LIST OF FIGURES	xiii
LIST OF ABBREVIATIONS	xv
CHAPTER I INTRODUCTION	1
1. Rationale and background	1
2. Research questions	3
3. Hypothesis of the study	3
4. Objective of the study	4
5. Scope and limitation of the study	4
6. Anticipated outcomes	5
CHAPTER II LITERATURE REVIEWS	7
1. Cholangiocarcinoma	8
1.1 Definition and pathology	8
1.2 Epidemiology	11
1.3 Etiology	12
1.4 Clinical manifestations	12
1.5 Treatments	13
2. Genetic alteration in cancer and cholangiocarcinoma	14
2.1 Genetic alterations and cancer development	14
2.2 Genetic alterations and cholangiocarcinoma	18

TABLE OF CONTENTS (Cont.)

	Page
3. Epigenetic alteration in cancer and cholangiocarcinoma	21
3.1 Epigenetics, DNA methylation and gene silencing	21
3.2 Epigenetics and application for cancer therapy	25
3.3 Hypermethylation of tumor suppressor genes and cancer	27
3.4 Hypermethylation of tumor suppressor genes and cancer	29
3.5 Techniques for detection of DNA methylation	31
CHAPTER III PART I: CpG-ISLAND METHYLATION PROFILING IN LIVER FLUKE RELATED CHOLANGIOCARCINOMA	43
1. Introduction	43
2. Materials and methods	45
2.1 CCA and adjacent normal samples	45
2.2 Clinicopathological data of CCA patients	45
2.3 DNA extraction from frozen tissues	46
2.4 Cell lines, cell culture and DNA extraction	46
2.5 <i>In vitro</i> methyltransferase treated placental DNA	47
2.6 Bisulfite modification	47
2.7 Detection of methylation status using methylation specific polymerase chain reaction	48
2.8 Pyrosequencing	53
2.9 Combined bisulfite restriction analysis	54
2.10 Protein expression analysis by immunohistochemistry	56
2.11 Statistic analysis	57

TABLE OF CONTENTS (Cont.)

	Page
3. Results	58
3.1 Frequency of CpG-island methylation in cholangiocarcinoma and adjacent normal tissues	58
3.2 Concurrent methylation of multiple CpG-islands in CCA	62
3.3 Correlation between CpG-island methylation and clinicopathological data	66
3.4 Validation of methylation status of candidate CpG-islands	69
3.5 Expression of OPCML and DcR1	70
4. Discussion	72
CHAPTER IV PART II: GENOME-WIDE DNA METHYLATION SIGNATURE IN CHOLANGIOCARCINOMA	77
1. Introduction	77
2. Materials and methods	78
2.1 Samples	78
2.2 Bisulfite conversion and Infinium Methylation Assay	78
2.3 Pyrosequencing	79
2.4 Statistical and bioinformatics analysis	82
3. Results	83
3.1 Methylation assay reproducibility	83
3.2 DNA methylation profiles in cholangiocarcinoma and normal	84
3.3 DNA methylation changes between primary CCA and adjacent normal samples	86
3.4 Specific functional terms of DNA methylation profiles	90

TABLE OF CONTENTS (Cont.)

	Page
3.5 Validation of HumanMethylation27 BeadChip by pyrosequencing	98
4. Discussion	100
CHAPTER V CONCLUSIONS AND STUDY REMARKS	103
REFERENCES	107
APPENDICES	127
APPENDIX A SAMPLE SIZE	129
APPENDIX B CLINICOPATHOLOGICAL DATA	133
APPENDIX C CHEMICALS AND REAGENTS	139
APPENDIX D REAGENTS FOR MSP ASSAY	143
APPENDIX E SUPPLEMENTARY DATA FOR MSP RESULTS	147
APPENDIX F SUPPLEMENTARY DATA FOR PYROSEQUENCING AND COBRA	151
APPENDIX G VALIDATION RESULTS OF METHYLATION STUDY IN <i>OPCML</i> , <i>SFRP1</i> , <i>HIC1</i> AND <i>DcR1</i>	157
APPENDIX H REAGENTS FOR IMMUNOHISTOCHEMISTRY	163
APPENDIX I IMMUNOHISTOCHEMISTRY AND METHYLATION RESULTS OF OPCML AND DcR1	167
APPENDIX J SUPPLEMENTARY DATA FOR GENOME-WIDE DNA METHYLATION STUDY (ILLUMINA STUDY)	173
APPENDIX K PYROSEQUENCING RESULTS OF HOXA9 AND HOXD9	185
APPENDIX L RESEARCH PUBLICATION AND PRESENTATION	191
CURRICULUM VITAE	199

LIST OF TABLES

	Page
Table 2-1 Histopathological classification of CCA	11
Table 2-2 Molecular aberrations of cholangiocarcinoma	20
Table 2-3 Examples of genes that can become epigenetically inactivated in tumors	28
Table 2-4 Epigenetic aberration of cholangiocarcinoma	30
Table 3-1 Sequences of methylated-specific primers for MSP with their annealing temperature (T_A), $MgCl_2$ concentration and PCR product sizes	49
Table 3-2 Primer sequences of PCR for COBRA of <i>OPCML</i> , <i>SFRP1</i> and <i>HIC1</i>	55
Table 3-3 PCR conditions for COBRA of <i>OPCML</i> , <i>SFRP1</i> and <i>HIC1</i>	55
Table 3-4 The conditions for digestion with restriction enzyme for COBRA analysis	56
Table 3-5 Methylation of selected methylated CpG-islands in CCA cell lines	61
Table 3-6 Association between methylation of gene profiling and clinicopathological data	68
Table 3-7 Validation of methylation status of <i>OPCML</i> using pyrosequencing and COBRA compared to MSP	69
Table 3-8 Validation of methylation status of <i>DcR1</i> using pyrosequencing compared to MSP	70
Table 3-9 Protein expression and methylation status of <i>OPCML</i>	71
Table 3-10 Protein expression and methylation status of <i>DcR1</i>	71

LIST OF TABLES (Cont.)

	Page
Table 4-1 Primer sequences and PCR product sizes of <i>HOXA9</i> and <i>HOXD9</i> for pyrosequencing	80
Table 4-2 PCR conditions for pyrosequencing of <i>HOXA9</i> and <i>HOXD9</i>	81
Table 4-3 Differentially methylated CpG sites normal and CCA samples	87
Table 4-4 Homeobox genes associated with differentially hypermethylated CpG sites within CpG-islands	91
Table 4-5 Gene Set Enrichment Analysis of hypermethylation compared between CCA and adjacent normal samples	95
Table 4-6 Gene Set Enrichment Analysis of hypomethylation compared between CCA and adjacent normal samples	96

LIST OF FIGURES

	Page
Figure 2-1 Macroscopic types of intrahepatic CCA	10
Figure 2-2 The proposed mechanism for pathogenesis of cholangiocarcinoma	19
Figure 2-3 Mechanism of DNA methylation	22
Figure 2-4 Proposed mechanism by which DNA methylation leads to transcriptional repression	24
Figure 2-5 DNA methyltransferase (DNMT) inhibition by enzyme trapping or enzyme blocking	26
Figure 2-6 Outline of the COBRA procedure	33
Figure 2-7 The workflow of bisulfite sequencing (BS)	35
Figure 2-8 The workflow of pyrosequencing	36
Figure 2-9 Diagram illustrating how to analyze DNA methylation by methylation specific oligonucleotide (MSO) array	38
Figure 2-10 Workflow of chromatin immunoprecipitation (ChIP) on DNA microarray (ChIP-chip) analysis	39
Figure 2-11 The Infinium assay for methylation study	41
Figure 3-1 Methylation profile of 26 CpG-islands in cholangiocarcinoma and adjacent normal samples	59
Figure 3-2 Frequency of methylation of 26 CpG-islands in adjacent normal samples and cholangiocarcinoma	60
Figure 3-3 Number of methylated CpG-island and methylation index in adjacent normal samples and cholangiocarcinoma	63
Figure 3-4 Concurrent methylation of highly methylated CpG-islands in cholangiocarcinoma	64
Figure 3-5 Methylation index between methylated and unmethylated status of 5 highly methylated CpG-islands	65
Figure 3-6 The association between methylation of <i>DcR1</i> and overall survival time of cholangiocarcinoma patients	67

LIST OF FIGURES (Cont.)

	Page
Figure 4-1 Methylation assay reproducibility	83
Figure 4-2 Hierarchical clustering of DNA methylation profiles	85
Figure 4-3 Differential DNA hypermethylated CpG sites within and out of CGIs between tumors and adjacent normal samples	88
Figure 4-4 Differential DNA hypomethylated CpG sites within and out of CGIs between tumors and adjacent normal samples	89
Figure 4-5 Boxplots represent differential methylation of <i>HOXA9</i> and <i>HOXD9</i> between adjacent normal and primary CCA detected by pyrosequencing	99
Figure 5-1 Scheme of the emergent integrated circuit of the cell	104
Figure 5-2 Scheme of the TRAIL signalling pathway	105

LIST OF ABBREVIATIONS

A	Adenine
ASIR	Age-standardized incidence rate
β	Beta
bp	Base pair
BS	Bisulfite sequencing
BSA	Bovine serum albumin
C	Cytosine
^m C	Methylcytosine
CCA	Cholangiocarcinoma
CGI	CpG-island
ChIP	Chromatin immunoprecipitation
°C	Degree Celsius
COBRA	Combined bisulfate restriction analysis
DAB	3, 3'-Diaminobenzidine tetrahydrochloride
ddNTPs	dideoxyribonucleotide triphosphate
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DNMT	DNA methyltransferase
DNP	2,4-Dinitrophenol
dNTPs	Deoxynucleoside triphosphates
ECC	Extrahepatic cholangiocarcinoma
EDTA	Ethylenediamine tetraacetic acid
EGF	Epidermal growth factor
ES	Embryonic stem cell
FDR	False discovery rate
5-FU	5-Fluorouracil

LIST OF ABBREVIATIONS (Cont.)

g	Gram
G	Guanine
h	Hour
HCC	Hepatocellularcarcinoma
HCl	Hydrochloric acid
HDAC	Histone deacetylase
HMT	Histone methyltransferase
ICC	Intrahepatic cholangiocarcinoma
ID	Intraductal growing type
IHC	Immunohistochemistry
kb	Kilobase
L	Liter
LOH	Loss of heterozygosity
μ l	Microliter
M	Molar
MF	Mass-forming type
MgCl ₂	Magnesium chloride
MI	Methylation index
μ M	Micromolar
ml	Milliliter
mM	Millimolar
min	Minute
mRNA	Messenger ribonucleic acid
MSI	Microsatellite instability
MSO	Methylation-specific oligonucleotide
MSP	Methylation-specific polymerase chain reaction

LIST OF ABBREVIATIONS (Cont.)

MS-SSCP	Methylation-specific single strand conformation polymorphism
NaCl	Sodium chloride
NaOH	Sodium hydroxide
ng	Nanogram
NO	Nitric oxide
OV	<i>Opisthorchis viverrini</i>
PBS	Phosphate buffer saline
PcG	Polycomb group
PCR	Polymerase chain reaction
PI	Periductal-infiltrating type
pmol	Picomole
PSC	Primary sclerosing cholangitis
RNA	Ribonucleic acid
T	Thymidine
T _A	Annealing temperature
TNM	Tumor-node-metastasis
TRAIL	TNF-related apoptosis-inducing ligand
TSA	Trichostatin
U	Uracil
UFT	Tegafur-Uracil
VPA	Vaproic acid
WGA	Whole-genome amplification