Aberrant methylation of *HTATIP2* and *UCHL1* as a predictive biomarker for cholangiocarcinoma

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Received August 25, 2017; Accepted December 6, 2017

DOI: 10.3892/mmr.2017.8319

Abstract. Cholangiocarcinoma (CCA) is the most common primary liver cancer in Northeastern Thailand where liver fluke infection is highly endemic. Although aberrant DNA methylation in CCA has been reported by several investigators, little is known regarding the associations between them. In the present study, the results obtained from our previously published methylation array were analyzed and 10 candidate genes involved in DNA repair [protein phosphatase 4 catalytic subunit (*PPP4C*)], apoptosis [runt related transcription factor 3 (*RUNX3*), interferon regulatory factor 4 (*IRF4*), ubiquitin C-terminal hydrolase L1 (*UCHL1*) and tumor protein p53 inducible protein 3 (*TP53I3*)], cell proliferation [cyclin D2 (*CCND2*) and Ras association domain family member 1 (*RASSF1*)], drug metabolism [aldehyde dehydrogenase 1 family member A3 (*ALDH1A3*) and solute carrier family 29 member 1 (*SLC29A1*)] and angiogenesis [human immunodeficiency virus-1 tat interactive protein 2 (*HTATIP2*)] were selected for quantification of their methylation levels in 54 CCA and 19 adjacent normal tissues using methylation-sensitive high-resolution melting. The associations between the methylation status of the individual genes and clinical parameters were statistically analyzed. High methylation levels were observed in *UCHL1*, *IRF4*, *CCND2*, *HTATIP2* and *TP53I3*. The median methylation level of *UCHL1* was 57.3% (range, 3.15 to 88.7%) and *HTATIP2* was 13.6% (range, 7.5 to 36.7%). By contrast, low methylation of *HTATIP2* and *UCHL1* was identified in adjacent normal tissues. The methylation status of *HTATIP2* and *UCHL1* was associated with patients' overall survival. CCA patients with high methylation of *HTATIP2* and low methylation of *UCHL1* exhibited longer overall survival. In addition, multivariate Cox regression analysis demonstrated that *UCHL1* methylation was an independent factor for CCA with hazard ratio of 1.81 (95% confidence interval, 1.01-3.25) in high methylation group. The combination of *HTATIP2* and *UCHL1* methylation status strongly supported their potential predictive biomarker in which patients with CCA who had high methylation of *HTATIP2* and low methylation of *UCHL1* showed longer overall survival than those with low *HTATIP2* methylation and high *UCHL1* methylation. In conclusion, the present study revealed the value of aberrant DNA methylation of *HTATIP2* and *UCHL1*, which may serve as a potential predictive biomarker for CCA.

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Key words: tumor biomarkers, biliary cancer, methylation-sensitive high-resolution melting, epigenetic, chemotherapy response

Introduction

Cholangiocarcinoma (CCA) is a malignancy of bile duct epithelium related with a high invasion and metastasis. CCA can be classified into perihilar/extrahepatic and intrahepatic type, based on anatomical location. The incidence and mortality rates of CCA, particularly the intrahepatic type, have been increasing worldwide (1-3). The prevalence of CCA in Northeast Thailand, especially in Khon Kaen, has been found about 89% of all primary liver cancers which is related with liver flukes [*Opisthorchis viverrini* (OV)] infection, making the region as the area of the highest incidence in the world (84 per 100,000 in men and 36 per 100,000 in women) (4-6). Prolonged parasitic infection possibly induces chronic inflammation of biliary tract to carcinogenic substances leading to genetic and epigenetic aberrations in the epithelial cells (7). The prognosis of patients with unrespectable tumors is poor, of which the overall survival rates are low and the majority of patients die within a year of diagnosis because no effective treatment is available (8,9). Luvira *et al* (8) found that the overall median survival of CCA patients in Northeast Thailand was 4 months (95% CI, 3.3-4.7) and 2-year survival rate was only 8.1% (95% CI, 4.5-12.9).

Our previous study on the genome-wide methylation using the Infinium HumanMethylation27 BeadChip microarray (Illumina, Inc., San Diego, CA, USA) (10) demonstrated hypo- and hyper-methylation status of 28 CCA cases in comparison

with 6 match normal adjacent tissues. The methylation level of individual genes obtained from methylation array data was presented as β-value ranging from 0 (unmethylation) to 1 (complete or 100% methylation). To address whether aberrant methylation of genes involved in chemotherapeutic response was different between short and long survival, in this study, 8 of 28 CCA cases treated with 5-fluorouracil (5-FU) consisting of 4 short and 4 long survival were selected for data analysis based on their differential β-values of genes which play roles in DNA repair, apoptosis, cell proliferation, drug metabolism and angiogenesis. Based on previous study for gene selection (11), differential β-value of >0.1 (10% fold-change) indicating 1.5 differential fold-change of gene expression was applied. Accordingly, 10 genes which had differential β-values ranging from 0.1-0.4 in short and long survival were selected. There were DNA repair: protein phosphatase 4 catalytic subunit (*PPP4C)* (12); apoptosis: runt related transcription factor 3 (*RUNX3)* (13,14), interferon regulatory factor 4 (*IRF4)* (15), ubiquitin C-terminal hydrolase L1 (*UCHL1)* (16), tumor protein p53 inducible protein 3 (*TP53I3)* (17); cell proliferation: cyclin D2 (*CCND2)* (18), Ras association domain family member 1 *(RASSF1)* (19); drug metabolism: aldehyde dehydrogenase 1 family member A3 *(ALDH1A3)* (20), solute carrier family 29 member 1 *(SLC29A1)* (21); and angiogenesis: human immunodeficiency virus-1 tat interactive protein 2 (*HTATIP2)* (22). RASSF1 plays roles in the apoptotic DNA damage response (DDR), cell proliferation and regulates XPA-mediated DNA repair (23), however, *RASSF1* was categorized into cell proliferation in this study. *RUNX3* and *IRF4* are transcription factor genes which play a role in apoptosis, they were categorized into apoptosis. These 10 genes were studied in more detail for their methylation status in CCA samples using methylation-sensitive high-resolution melting (MS-HRM). Moreover, their association with clinicopathological data was also analyzed.

Materials and methods

Samples and DNA preparation. Fifty-four frozen liver tissues of intrahepatic CCA patients with clinicopathological data and 19 matched adjacent normal samples were kindly supplied by the Cholangiocarcinoma Research Institute, Khon Kaen University, Khon Kaen, Thailand. Written informed consent was obtained from all patients. The project was approved by the Khon Kaen University Ethics Committee for Human Research (HE571022).

DNA methylation levels of 10 candidate genes were quantified in 54 CCA patients that were then divided into two groups as low and high methylation based on a cut-off value (median of methylation level of individual genes) for survival analysis. The survival time is the time after operation date until the date of death. Genes which were significantly correlated with survival time were further performed for survival analysis in 42 of 54 CCA cases that underwent 5-FU therapy after surgery (12 were excluded as untreated patients). Of 42 5-FU treated cases, 19 normal adjacent tissues were obtained and quantified for methylation levels which were used to compare to those of 42 tumor tissues.

DNA from frozen liver tissues was extracted using Qiagen® Blood & Tissue kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. Normal male leukocyte genomic DNA was used as an unmethylated control. For *in vitro* methylated DNA, 10 μg of male leukocyte genomic DNA was treated with 10 units of SssI methyltransferase (New England Biolabs, Ipswich, MA) containing 160 μM S-adenosylmethionine and 1x methylase buffer at 37˚C for 1 h. The mixture was incubated at 65˚C for 20 min to stop reaction, followed by ethanol precipitation and resuspended in sterile water. Methylated and unmethylated DNA controls were spectroscopically quantified using the NanoVue (GE Healthcare, Buckinghamshire, UK) and stored at -20˚C until bisulfite modification.

Bisulfite modification. Sodium bisulfite modification was performed using the EZ DNA Methylation-Gold kit (Zymo Research Corp., Irvine, CA, USA) following the manufacturer's instructions. The eluted DNA was ethanol precipitated and dissolved into 20 μl of distilled water, after which was spectroscopically quantified at 260 nm using the NanoVue (GE Healthcare). Complete bisulfite modified DNA was validated using modified and wild type *Calponin* specific primers as described previously (24). Sample which was not found a specific band from modified primer (333 bp) and/or given a specific band with wild type primers (333 bp) was considered unmodified or incompletely modified. Only complete bisulfite modified DNA was used as a DNA template for PCR and MS-HRM assay in this study.

Primers. Primers were designed and examined via electronic PCR (ePCR) using Methyl Primer Express program v1.0 (Applied Biosystems; http://bisearch.enzim.hu/?m=genompsearch). PCR conditions were optimized which could amplify both unmethylated and methylated DNA at the specific CpG islands. The primer sequences of 10 candidate genes including optimal conditions for MS-HRM are shown in Table I. The two different HTATIP2 isoforms are encoded by the same promotor. The alternative splicing is taken place at the C-terminus, as a result, two isoforms are generated. In this study, we performed DNA methylation at the gene promotor of *HTATIP2*. All primers were supplied by Pacific Science Co. Ltd., Bangkok, Thailand.

MS-HRM. MS-HRM can detect both methylated and unmethylated nucleotide difference due to different melting temperature by using a single primer set. The HRM can determine change of decreasing fluorescence intensity of PCR products with DNA duplex melting temperature (melting temperature in CG-rich product is higher than that of AT-rich product). Therefore, MS-HRM consists of real-time PCR using bisulfite-converted DNA and melting analysis of PCR products (HRM) which reflects the thermodynamic behavior of the MS-HRM amplicon. As a result, the methylation status can be determined through comparison with melting standard curves created by different dilution ratios of methylated and unmethylated DNA controls (25).

PCR amplification and HRM were performed on LighCycler480® Real time PCR machine (Roche, Mannheim, Germany). The 20 μl of PCR reaction consisted of 1x PCR buffer (67 mM Tris, Ph 8.4, 16.6 mM ammonium sulfate and 0.1% Tween-20), 300 nM of each primer, 200 μM of each dNTP,

Table I. Primer sequences for methylation-sensitive high-resolution melting, optimized conditions and product details.

Primer name	Sequences (5'-3')	Ta (˚C)	$MgCl_2$ (mM)	Product	
				Size (bp)	CG site
UCHL1-F	CGAGTGAGATTGTAAGGTTTGG	55	2.5	143	14
UCHL1-R	AACGCACTATAAAACCTATACAA				
IRF4-F	GCGTTGGTTTGGGTTTTAA	58	2.5	143	13
IRF4-R	CCGCCTCAACCACTCCT				
CCND2-F	GAAGCGAGGTTGTTTTGG	58	3.5	159	15
CCND2-R	CCCCGACTCTCTTCCTAAC				
HTATIP2-F	TTCGATTAGGGAAGGTGGGA	56	2.5	176	15
HTATIP2-R	CCGACCAAAAAACCTAACC				
TP53I3-F	GTTTCGTTGTTTTGGTTTGT	53	3.5	145	14
TP53I3-R	TACCGCATCCAACCCTAT				
RUNX3-F	AACGTTTGGAGAGTAGTGTT	55	2.5	139	14
RUNX3-R	CGATAAAAAAACCTACCCTC				
RASSF1-F	GGTTTCGGTATTTAGTATTTAGG	57	3.5	120	12
RASSF1-R	ATCGATAAACAACCCCACCC				
ALDH1A3-F	GGCGAAGTTTTAGGGTTT	56	3.5	147	17
ALDH1A3-R	CACGTACCCTACTCTTAAATC				
PPP4C-F	GGTCGATGTGAGGGGAGG	60	2.5	130	13
PPP4C-R	CACCGCACAAAAATCTCCTAA				
SLC29A1-F	TTCGGGTTTAAAATAGGTTG	57	2.5	155	13
SLC29A1-R	CGAACCTCCATCCCCATC				

F, forward; R, reverse; Ta, annealing temperature; UCHL1, ubiquitin C-terminal hydrolase L1; IRF4, interferon regulatory factor 4; CCND2, cyclin D2; HTATIP2, human immunodeficiency virus-1 tat interactive protein 2; TP53I3, tumor protein p53 inducible protein 3; RUNX3, runt related transcription factor 3; RASSF1, Ras association domain family member 1; ALDH1A3, aldehyde dehydrogenase 1 family member A3; PPP4C, protein phosphatase 4 catalytic subunit; SLC29A1, solute carrier family 29 member 1.

20 ng of bisulfite modified DNA, 1.5 μM SYTO®9 (Invitrogen, Carlsbad, CA), 0.5 unit of Platinum *Taq* DNA polymerase (Invitrogen), and $MgCl_2$ concentration as described in Table I. The cycling stage was performed as follows: Holding at 95˚C for 10 min to activate enzyme, 40 cycles of denaturation at 95˚C for 15 sec, annealing at temperature as described in Table I for 30 sec and extension at 72˚C for 1 min. After amplification, HRM stage was initiated by denaturing PCR product at 95˚C for 1 min, followed by reannealing at 40˚C for 1 min and slowly warmed by continuous acquisition to 95˚C with 1% ramp rate (˚C/s). All DNA samples were analyzed on a Light Cycler 480® 8-Tube strips with Light Cycler 480® sealing foil (Roche). Each reaction was performed in triplicate and no DNA template control was included in each experiment. To quantify DNA methylation of each assay, a standard curve was generated by running MS-HRM of standard dilution series including 100, 50, 25, 10, 5, 1 and 0% methylated controls. The precision of the assay was performed by including internal controls (25 and 75% methylated DNA) in each PCR run. The amplification plot and raw data of MS-HRM were reviewed using The Light Cycler 480®. MS-HRM data was analyzed using The Light Cycler 480® Gene Scanning Software. The calibration curve and linear equation of each MS-HRM were performed in Microsoft Excel 2016 and used for determination of methylation level of individual genes in clinical samples.

Statistical analysis. The correlations of clinicopathological data of CCA patients including age at initial diagnosis, sex, histological types, 5-FU treatment and the postoperative survival time with methylation status of individual genes were analyzed using Fisher's exact test. The difference of methylation levels between two independent groups was analyzed using Mann-Whitney U test, and Wilcoxon Matched-Pairs Signed-Ranks test was used for matched pair samples. Overall survival was analyzed using Kaplan-Meier and log-rank test, the univariate and multivariate Cox regression models. The statistical analysis was performed using SPSS version 17.0 for windows (SPSS Inc., Chicago, IL, USA). $P<0.05$ was considered to indicate a statistically significant difference.

Results

Quantification of DNA methylation by MS-HRM. MS-HRM of individual genes was performed using standard dilution series including 100, 50, 25, 10, 5, 1 and 0% methylated controls. The lower detection limit of most genes was 5% except 1% for *UCHL1*. The MS-HRM representatives of *HTATIP2* and *UCHL1* are shown in Fig. 1. The reproducibility of MS-HRM was performed using internal controls (25 and 75% of methylated DNA in a background of unmethylated DNA) which were run in triplicate in every experiment. The coefficient of variation (%CV) of both intra- and inter-assay of this study was less than 10%.

Aberrant DNA methylation is commonly found in CCA. DNA methylation levels of 10 candidate genes were quantified in

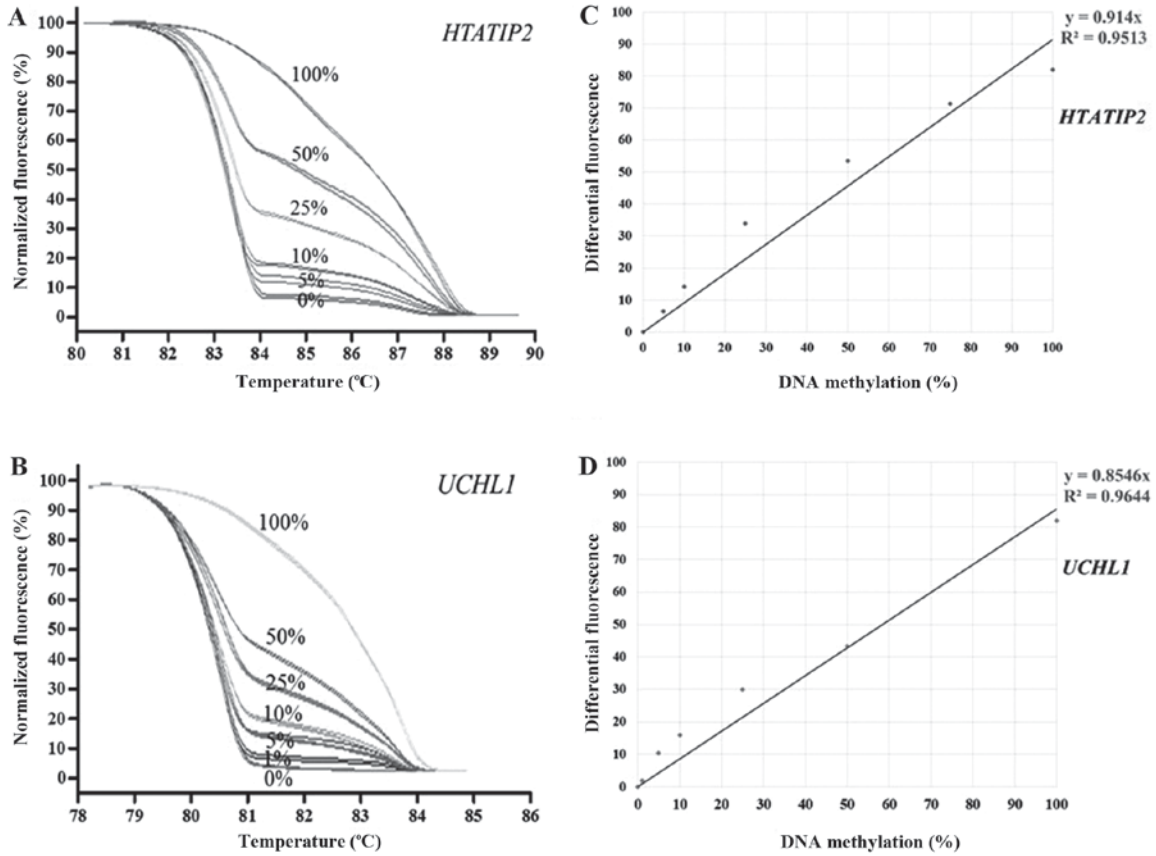

Figure 1. Methylation-sensitive high-resolution melting of *HTATIP2* and *UCHL1*. Aligned melt curves of (A) *HTATIP2* and (B) *UCHL1*. The plot between differential fluorescence and percentage of methylation (0, 1, 5, 10, 25, 50 and 100% methylation) of (C) *HTATIP2* and (D) *UCHL1*. HTATIP2, human immunodeficiency virus-1 tat interactive protein 2; UCHL1, ubiquitin C-terminal hydrolase L1.

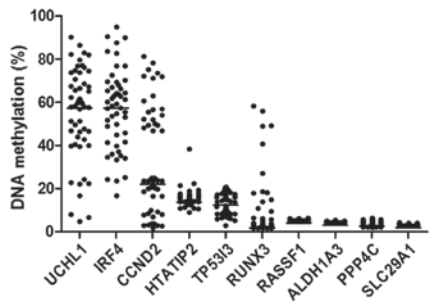

Figure 2. Scatter plot with median line graphics of the DNA methylation levels of the 10 candidate genes in the 54 cholangiocarcinoma samples. UCHL1, ubiquitin C-terminal hydrolase L1; IRF4, interferon regulatory factor 4; CCND2, cyclin D2; HTATIP2, human immunodeficiency virus-1 tat interactive protein 2; TP53I3, tumor protein p53 inducible protein 3; RUNX3, runt related transcription factor 3; RASSF1, Ras association domain family member 1; ALDH1A3, aldehyde dehydrogenase 1 family member A3; PPP4C, protein phosphatase 4 catalytic subunit; SLC29A1, solute carrier family 29 member 1.

54 CCA samples using MS-HRM. As shown in Fig. 2, the distribution of DNA methylation levels showed high percentage in *UCHL1* and *IRF4* with median of 57.29 and 56.32%, respectively. The moderate DNA methylation levels were found in *CCND2*, *HTATIP2* and *TP53I3* (median; 22.02, 13.59 and 12.41%, respectively), whereas *RUNX3*, *RASSF1*, *ALDH1A3*, *PPP4C* and *SLC29A1* had very low DNA methylation levels (median; 1.69, 4.11, 3.05, 2.63 and 1.87%, respectively).

DNA methylation of HTATIP2 and UCHL1 was associated with patients' overall survival. According to the median of methylation level of each candidate gene, 54 CCA patients were divided into two groups, depending on their methylation level, as low and high methylation level groups. The median methylation level of high and low *HTATIP2* methylation group was 15.10 and 12.31%, respectively. The median methylation level of high and low *UCHL1* methylation group was 69.10 and 38.69%, respectively. Our results showed that CCA patients with high *HTATIP2* methylation had significantly longer overall survival than those with low methylation (median; 59.4 vs. 26.2 weeks, P=0.003) (Fig. 3A). In contrast, CCA patients who had low *UCHL1* methylation showed significantly longer overall survival than those who had high methylation (median; 61.3 vs. 33.3 weeks, P=0.044) (Fig. 3B). Moreover, the multivariate Cox regression analysis demonstrated that *UCHL1* was an independent factor for CCA with hazard ratio of 1.81 (95%CI, 1.01-3.25) in high methylation group (Table II). However, no significant differences in correlation between DNA methylation and overall survival time of *CCND2*, *PPP4C*, *IRF4*, *ALDH1A3*, *SLC29A1*, *TP53I3*, *RASSF1*, and *RUNX3* were found. Also, no significant correlations of DNA methylation status of individual gens with age, sex, histological type, tumor stage and chemotherapy were observed.

Of 54 CCA, only 42 cases underwent 5-FU chemotherapy after surgery and were divided into two groups, depending on their methylation level, as low and high methylation

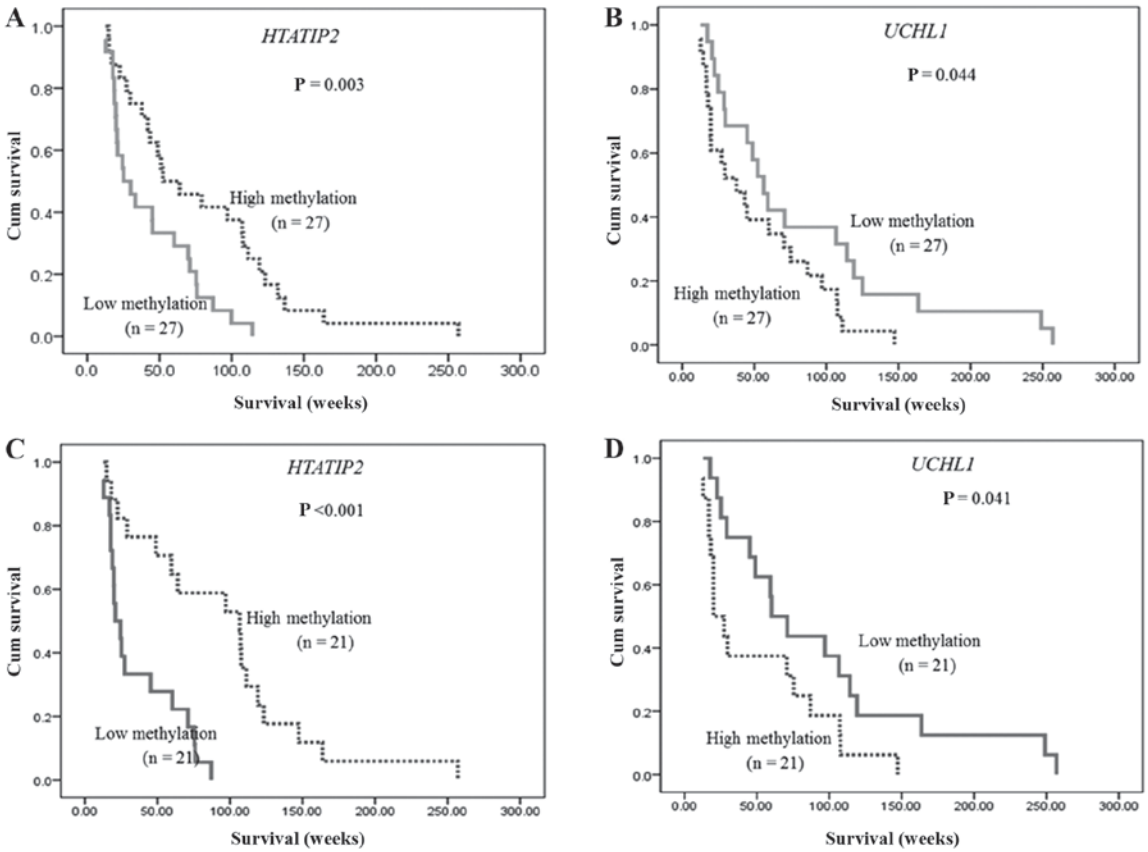

Figure 3. Kaplan-Meier curves of patients with CCA. CCA patients with (A) high methylation of *HTATIP2* and (B) low methylation of *UCHL1* showed longer overall survival. Similar results were obtained in the 42 5-fluorouracil treated patients with CCA, in which good prognosis was observed in patients who had (C) high methylation of *HTATIP2* and (D) low methylation of *UCHL1*. CCA, cholangiocarcinoma; HTATIP2, human immunodeficiency virus-1 tat interactive protein 2; UCHL1, ubiquitin C-terminal hydrolase L1; Cum, cumulative.

level groups; according to the median of methylation level of *HTATIP2* and *UCHL1* (median; 13.41 and 58.03%, respectively). The Kaplan-Meier analysis of 5-FU treated CCA patients showed that patients with high methylation of *HTATIP2* and low methylation of *UCHL1* had longer overall survival (83.8 vs. 22.7 weeks, P<0.001; 65.6 vs. 25.7 weeks, P=0.041, respectively) as shown in Fig. 3C and D, respectively. Nevertheless, no significant differences in correlation between DNA methylation and overall survival time of *CCND2, PPP4C, IRF4, ALDH1A3, SLC29A1, TP53I3, RASSF1*, and *RUNX3* were found.

In addition, the combination of *HTATIP2* and *UCHL1* methylation status exhibited that CCA patients who had both high methylation of *HTATIP2* and low methylation of *UCHL1* showed significantly longer overall survival time than those with both low methylation of *HTATIP2* and high methylation of *UCHL1* (median; 70.0 vs. 27.3 weeks, P=0.010) (Fig. 4) indicating their potential as a predictive biomarker for CCA.

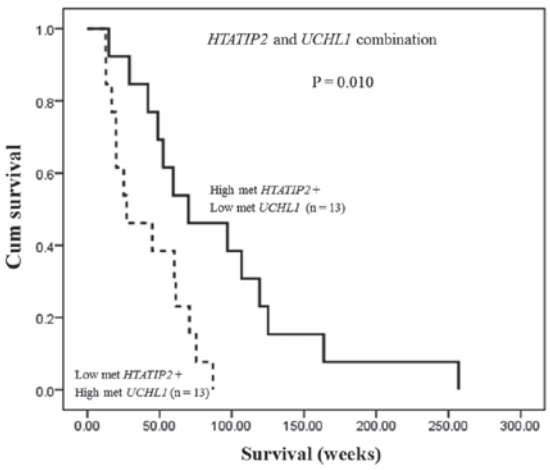

Figure 4. Kaplan-Meier curves of combined *HTATIP2* and *UCHL1* methylation. CCA patients with a combination of high *HTATIP2* methylation and low *UCHL1* methylation (n=13) exhibited longer overall survival than those with a combination of low *HTATIP2* methylation and high *UCHL1* methylation (n=13). CCA, cholangiocarcinoma; HTATIP2, human immunodeficiency virus-1 tat interactive protein 2; UCHL1, ubiquitin C-terminal hydrolase L1; Cum, cumulative.

High methylation of HTATIP2 and UCHL1 was found in CCA but not in adjacent normal tissue. To address whether high methylation levels of *UCHL1* and *HTATIP2* found in 42 CCA patients treated with 5-FU were not found in normal tissue, the methylation levels of these genes were determined in 19 adjacent normal tissues of 42 cases. As shown in Fig. 5A and B, the methylation levels of *HTATIP2* and *UCHL1* were significantly higher in CCA than in adjacent normal tissues. Matched-pair analysis of 19 cases showed that methylation levels of *HTATIP2* and *UCHL1* were significantly higher in CCA than in adjacent normal tissues (Fig. 5C and D).

Table II. Cox regression analysis of cholangiocarcinoma clinicopathological parameters and the DNA methylation of 10 genes.

	Univariate		Multivariate	
Parameters (n)	HR (95% CI)	P-value	HR (95% CI)	P-value
Age, years				
≤59 (26)	Reference			
>59 (28)	0.951 (0.55-1.65)	0.857	-	-
Sex				
Male (33)	Reference			
Female (21)	1.14 (0.65-1.99)	0.651	-	-
Histopathology				
Well differentiated (20)	Reference			
Less differentiated (11)	0.75 (0.33-1.69)	0.483	-	-
Staging				
I-II (4)	Reference			
III-IV (36)	0.87 (0.30-2.48)	0.794	-	-
Chemotherapy				
Treatment (42)	Reference			
No treatment (12)	0.70 (0.36-1.36)	0.289	-	-
UCHL1				
Low methylation (27)	Reference		Reference	
High methylation (27)	1.84 (1.03-3.29)	0.040	1.81 (1.01-3.25)	0.048
IRF4				
Low methylation (27)	Reference			
High methylation (27)	1.24 (0.72-2.13)	0.448	-	-
CCND2				
Low methylation (27)	Reference			
High methylation (27)	0.77 (0.44-1.33)	0.342	-	-
HTATIP2				
Low methylation (27)	Reference			
High methylation (27)	0.55 (0.32-0.97)	0.039	NS	NS
TP53I3				
Low methylation (27)	Reference			
High methylation (27)	0.92 (0.53-1.58)	0.755	-	-
RUNX3				
Low methylation (27)	Reference			
High methylation (27)	0.55 (0.31-0.99)	0.044	NS	NS
RASSF1				
Low methylation (27)	Reference			
High methylation (27)	1.03 (0.59-1.80)	0.911	-	-
ALDH1A3				
Low methylation (27)	Reference			
High methylation (27)	0.57 (0.32-0.99)	0.046	NS	NS
PPP4C				
Low methylation (27)	Reference			
High methylation (27)	1.36 (0.79-2.37)	0.268	-	-
SLC29A1				
Low methylation (27)	Reference			
High methylation (27)	1.02 (0.59-1.76)	0.939	-	-

A significant P<0.05 for each variable obtained from univariate analysis was selected for multivariate analysis using Cox regression, the backward stepwise method. Reference refers to the parameter used as a baseline for comparison. HR, hazard ratio; CI, confidence interval; NS, not significant; -, not included in multivariate analysis; *UCHL1*, ubiquitin C-terminal hydrolase L1; *IRF4*, interferon regulatory factor 4; *CCND2*, cyclin D2; *HTATIP2*, human immunodeficiency virus-1 tat interactive protein 2; *TP53I3*, tumor protein p53 inducible protein 3; *RUNX3*, runt related transcription factor 3; *RASSF1*, Ras association domain family member 1; *ALDH1A3*, aldehyde dehydrogenase 1 family member A3; *PPP4C*, protein phosphatase 4 catalytic subunit; *SLC29A1*, solute carrier family 29 member 1.

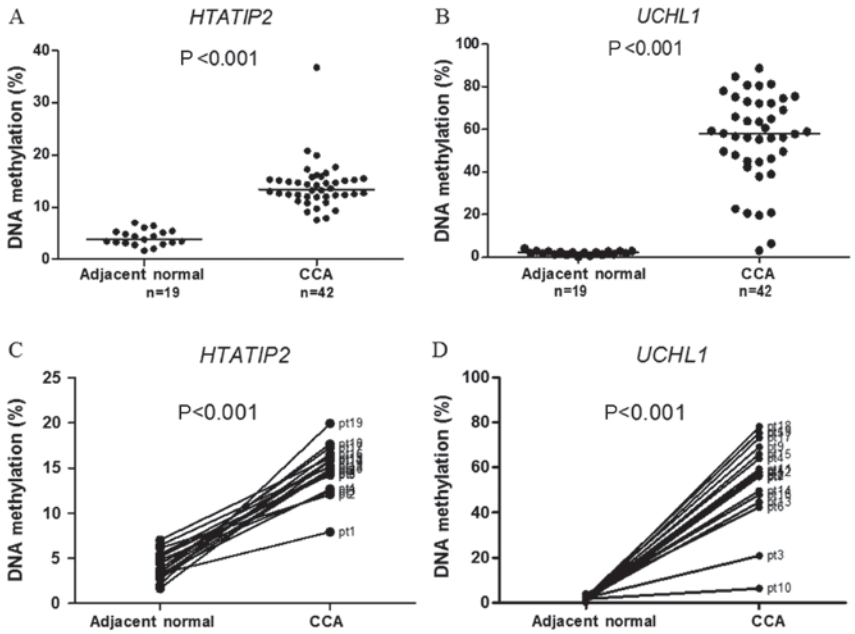

Figure 5. Scatter plots with median line graphics of DNA methylation levels of (A) *HTATIP2* and (B) *UCHL1* in adjacent normal tissues (n=19) compared with 5-fluorouracil treated CCA (n=42). Median methylation levels of *HTATIP2* and *UCHL1* were significantly higher in CCA than those in adjacent normal tissues (Mann-Whitney U test). Matched-pair analysis of methylation levels of (C) *HTATIP2* and (D) *UCHL1* in CCA and adjacent normal tissues (n=19). The methylation levels of the two genes were significantly higher in CCA than in adjacent normal tissues (Wilcoxon Matched-Pairs Signed-Ranks Test). CCA, cholangiocarcinoma; HTATIP2, human immunodeficiency virus-1 tat interactive protein 2; UCHL1, ubiquitin C-terminal hydrolase L1; pt, patient.

Discussion

Our results showed high methylation levels of multiple genes including *UCHL1*, *IRF4*, *CCND2*, *HTATIP2* and *TP53I3* while very low methylation was found in *RUNX3*, *RASSF1*, *ALDH1A3*, *PPP4C* and *SLC29A1*. This finding suggested that DNA methylation is an epigenetic event commonly found in CCA. However, we did not perform immunostaining of the proteins of these genes in CCA samples which could indicate the effect of DNA methylation on gene silencing. It is conceivable that DNA methylation of these genes, at least in part, may potentially lead to gene silencing which may have the great impact on cancer cell survival and progression. Patients treated with 5-FU in our pilot study (10) were divided into short and long survival to select genes differentially methylated, by which *HTATIP2* was one of 10 genes selected. In this present study, patients with high and low methylated *HTATIP2* were statistically analyzed with overall survival.

The present study showed that hypermethylation of two genes, namely *HTATIP2* and *UCHL1*, was not only tumor specific, but also correlated with patients' overall survival time. An important corollary of this study is the potential of methylation status of *HTATIP2* and *UCHL1* which can serve as predictive biomarkers for CCA in which *HTATIP2* hypermethylation is a favorable predictive marker, whereas *UCHL1* hypermethylation is an unfavorable predictive marker, and when used in combination may strengthen their potential.

HTATIP2 is implicated in the regulation of tumor cell growth, metastasis, apoptosis and autophagy related pathways (26,27). There are 2 isoforms of HTATIP2, in which isoform 1 is a metastasis suppressor with proapoptotic as well as anti-angiogenic properties which is oxidoreductase required for tumor suppression (26,28). However, small isoform 2 (TC3), 15 kDa, actually has a death-protective function (29). Hypermethylation of *HTATIP2* was found in 47% of hepatocellular carcinoma samples but was not detected in normal liver tissues (30). Another study showed that 36% of colorectal carcinoma samples had hypermethylated *HTATIP2* (31), whereas hypermethylation of *HTATIP2* was found in 98% (53/54) of CCA in this study. The high frequency of *HTATIP2* and *UCHL1* hypermethylation found in liver fluke related CCA may be due to chronic inflammation caused by liver fluke infection. It has been shown that DNA methylation is observed during inflammation and inflammation-associated carcinogenesis (32). Methylation of both genes may potentially lead to gene silencing resulting in no cell growth inhibition and apoptosis induction.

The UCHL1 is a de-ubiquitinating enzyme which cleaves, recycles ubiquitin molecules, and stabilizes ubiquitin pool suggesting its functions as tumor suppressor. UCHL1 has been reported to directly interact with p53 and stabilize p53 by inhibiting its degradation through the ubiquitination pathway (33). Restoration expression of silenced UCHL1 of hepatocellular carcinoma cell lines significantly inhibited cell growth and colony formation (33). *UCHL1* gene silencing by promoter methylation may deregulate the p53 pathway by reducing its ability in apoptosis induction caused by a chemotherapeutic agent in gastric cancer, by which UCHL1 re-expression could induce apoptosis (34). In the present study, *UCHL1* hypermethylation was observed in CCA, but not in the adjacent normal epithelium. We speculate that loss of UCHL1 expression due to promoter hypermethylation may result in increased p53 degradation via ubiquitination pathway, and consequently loss of p53 function in apoptosis induction.

This may in turn lead to acquired chemotherapeutic drug resistance in CCA. However, further study of UCHL1 in CCA is required in order to know its function which may be relevant to chemotherapy response.

We demonstrated that CCA patients with high methylation level of *HTATIP2* and low methylation level of *UCHL1* were associated with longer overall survival. This finding showed the significant association not only in 54 CCA but also in 42 5-FU treated CCA suggesting the great value of *UCHL1* and *HTATIP2* methylation as a predictive biomarker for CCA. Based on median overall survival of CCA patients in Northeast Thailand reported by Luvira *et al* (8) which was 4 months, it is hard to get the data of disease-free survival. The meta-analysis of 14 studies with 1705 patients involved revealed significant association between high expression of HTATIP2 in cancer patients and a good overall survival (35). In contradictory to our results, the methylation of the *HTATIP2* promoter did not significantly influence the overall survival.

Moreover, downregulation of HTATIP2 is involved in progression and aggressiveness of hepatocellular carcinoma (36) and decreased HTATIP2 expression in laryngeal carcinoma is related to poor prognosis and shorten survival (37), suggesting a tumor suppressive function of HTATIP2. Previous study showed that HTATIP2 might be an upstream regulator of p53 which induces apoptosis through both p53-dependent and -independent pathways (38), while mutant HTATIP2 down-regulates the endogenous expression of p53 mRNA and protein (39). Furthermore, HTATIP2 could reduce the expression of vascular endothelial growth factor (VEGF) mRNA which resulted in inhibition of angiogenesis (26). Dong *et al* (40) studied *HTATIP2* methylation which the CpG sites are the same as our study and protein expression in glioma patients and observed no significant association indicating that DNA methylation is not a major epigenetic event for gene silencing. Accordingly, high methylation level of *HTATIP2* may not reflect low protein expression in CCA patients. However, further study is required to precisely identify its roles in CCA.

Bonazzi *et al* (41) showed that hypermethylation of *UCHL1* is crucial for gene silencing in melanoma, of which the CpG sites are the same as our study. Poor prognosis was observed in CCA patients with high methylation of *UCHL1* implicating low expression of UCHL1. The expression of UCHL1 could activate the p14ARF-p53 signaling pathway by deubiquitinating p53 and p14ARF as well as ubiquitinating MDM2 through its two opposing enzyme activities, hydrolase and ligase (42,43). In opposite to our finding, high UCHL1 expression was significantly associated with shorter overall survival in breast cancer (44).

Although *IRF4*, *CCND2*, *TP53I3* exhibited high methylation levels in CCA patients, no correlation of their methylation status with patients' survival and clinical parameters was found. In addition, hypermethylation of *RUNX3* (45), *RASSF1* (46) and *ALDH1A3* (47) was found in multiple tumors such as meningiomas, gliomas and glioblastoma, but in our study, they exhibited very low methylation levels which were the same levels of normal epithelial cells.

In summary, our findings provide an insight into aberrant DNA methylation of *UCHL1*, *IRF4*, *CCND2*, *HTATIP2* and *TP53I3* as an epigenetic event of CCA. The methylation of *HTATIP2* and *UCHL1* can served as a potential predictive biomarker for CCA.

Acknowledgements

This project was supported by the Higher Education Research Promotion and National Research University Project of Thailand, the Office of the Higher Education Commission, through the Center of Excellence in Specific Health Problems in Greater Mekong Sub-region cluster (SHeP-GMS), Khon Kaen University, Thailand. C.N. was financially supported by the Centre for Research and Development of Medical Diagnostic Laboratories, Faculty of Associated Medical Sciences, Khon Kaen University, Khon Kaen, Thailand. We thank the Cholangiocarcinoma Research Institute, Khon Kaen University, Khon Kaen, Thailand for providing samples and clinical data.

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