รายงานวิจัยฉบับสมบูรณ์

โครงการ การพิสูจน์ตัวบ่งชี้เมททิลเลชั่นที่จำเพาะต่อเซลล์ตับของดีเอนเอในกระแสเลือดของผู้ป่วยโรคตับ

โดย ดร. นริศร คงรัตนโชค และคณะ

กันยายน 2563 ที่เสร็จโครงการ

สัญญาเลขที่ TRG5780181

รายงานวิจัยฉบับสมบูรณ์

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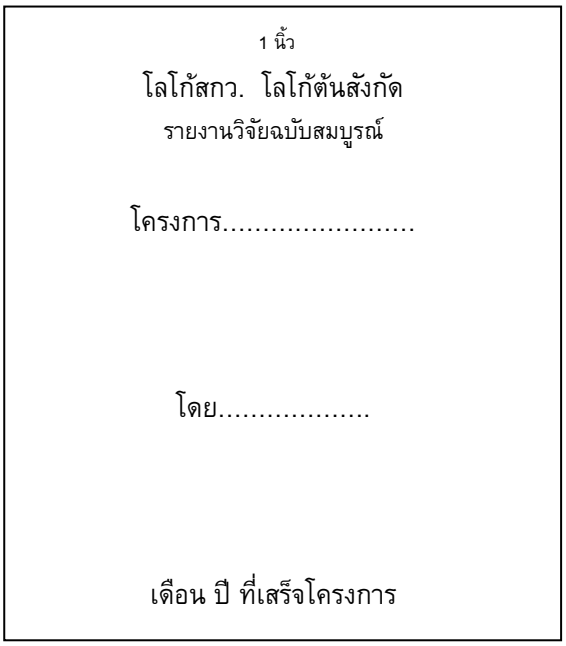
รูปแบบปกรายงานฉบับสมบูรณ์

ปกนอก



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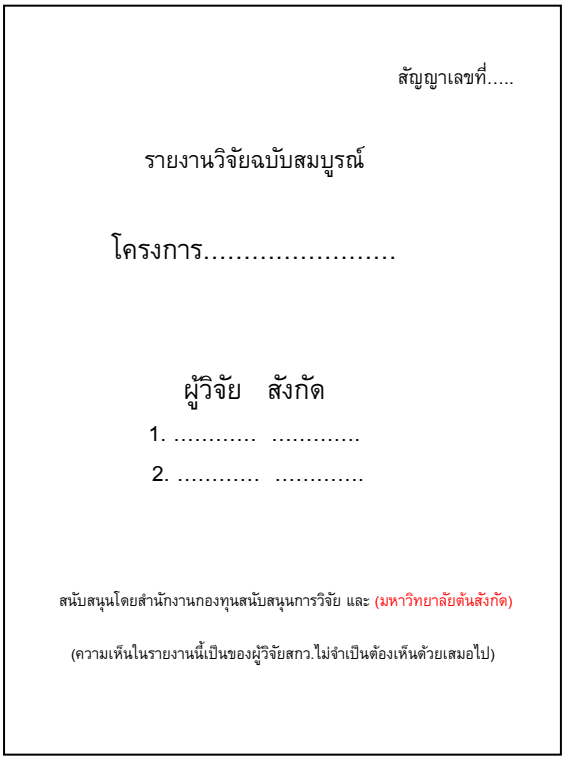

บทคัดย่อ

รหัสโครงการ: TRG5780181

ชื่อโครงการ: การพิสูจน์ตัวบ่งชี้เมททิลเลชั่นที่จำเพาะต่อเซลล์ตับของดีเอนเอในกระแสเลือดของผู้ป่วยโรคตับ

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บทคัดย่อ:

ความเป็นมา: การเปลี่ยนแปลงของภาวะเหนือพันธุกรรมของดีเอนเอในเลือดผู้ป่วยถูกวิเคราะห์เพื่อใช้ในการวินิจฉัย และการการพยากรณ์ของโรค ระดับดีเอ็นเอเมทิลเลชันของยีน SHP1P2 ถูกนำมาใช้เพื่อเป็นตัวบ่งชี้เฉพาะเจาะจงของเยื่อบุผิว สำหรับตัวบ่งชี้เฉพาะเจาะจงของตับเราทำการตรวจสอบความถูกต้องของระดับดีเอ็นเอเมทิลเลชันของยีน DSU ก่อน จากนั้นเราจึงตรวจสอบว่าระดับดีเอ็นเอเมททิลเลชันของยีน DSU และ SHP1P2 สามารถนำมาใช้เป็นตัวบ่งชี้ภาวะเหนือพันธุกรรมสำหรับตรวจวินิจฉัย และการการพยากรณ์ของโรคตับได้หรือไม่

วิธีการ: ไพโรซีเควนซิ่งถูกนำมาใช้เพื่อระบุรูปแบบเมทิลเลชันของยีน DSU โดยการเปรียบเทียบระดับเมทิลเลชัน ระหว่างเนื้อเยื่อปกติชนิดต่างๆ เซลล์ไลน์ชนิดต่างๆ และ เนื้อเยื่อมะเร็งของตับและของท่อน้ำดี สำหรับ เทคนิคเรียลไทม์พีซีอาร์ถูกนำมาใช้เพื่อตรวจหาปริมาณระดับดีเอ็นเอเมทิลเลชันของยีน DSU และ SHP1P2 ในพลาสมาของผู้ป่วยโรคตับ (โรคตับอักเสบบีเรื้อรัง โรคตับแข็ง และ มะเร็งตับ) และผู้ที่มีสุขภาพดี

ผล: ระดับเมทิลเลชันสูงของยีน DSU พบได้เฉพาะในเนื้อเยื่อตับปกติและเซลล์ตับ นอกจากนั้นเนื้อเยื่อของตับปกติและของมะเร็งตับมีระดับเมทิลเลชันสูงกว่าเนื้อเยื่อของมะเร็งท่อน้ำดีอย่างมีนัยสำคัญทางสถิติ ($P < 0.001$ และ $P < 0.05$ ตามลำดับ) ระดับเมทิลเลชันของยีน DSU และ SHP1P2 ในผู้ป่วยมะเร็งตับมีค่าสูงกว่าผู้ที่มีสุขภาพดีอย่างมีนัยสำคัญทางสถิติ ($P = 0.045$ และ $P < 0.001$ ตามลำดับ) ระยะของโรคและอัตราการรอดชีวิตมีความสัมพันธ์กับระดับเมทิลเลชันของ SHP1P2 ที่สูงขึ้น ($P < 0.001$ และ $P = 0.018$ ตามลำดับ) แต่ไม่มีความสัมพันธ์กับยีน DSU

สรุป: ยีน SHP1P2 เป็นตัวบ่งชี้ภาวะเหนือพันธุกรรมสำหรับการตรวจวินิจฉัยมะเร็งตับที่ดีกว่ายีน DSU อย่างไรก็ตามยีน DSU มีความเป็นไปได้ที่จะใช้เป็นตัวบ่งชี้ทางนิติวิทยาศาสตร์สำหรับการสืบสวนอาชญากรรม เนื่องจากเป็นตัวบ่งชี้เมทิลเลชันดีเอ็นเอที่จำเพาะต่อตับ

คำหลัก : Epigenetic marker, hepatocellular carcinoma, tissue specific DNA methylation, Circulating DNA

Abstract

Project Code : TRG5780181

Project Title : The validation of hepatocyte-specific DNA methylation marker in circulating nucleic acids of patient with liver disease

Investigator : Narisorn Kongruttanachok

E-mail Address : Ruttiwan@gmail.com

Project Period : 2 June 2014 to 1 June 2016

Abstract:

Background: Epigenetic alterations of circulating DNA are detected in patients for diagnosis and progression of diseases. *SHP1P2* genes was utilized to be an epithelial-specific DNA methylation marker. For a hepatocyte-specific DNA methylation marker, we was validated methylation profile of *DSU* gene. Then, we investigated whether *DSU* and *SHP1P2* genes could be non-invasive epigenetic markers for liver diseases.

Method: Pyrosequencing was used to identify the methylation patterns of *DSU* gene by comparing the methylation levels among different normal tissue types, different cell lines and tissue samples of HCC and cholangiocarcinoma. Tissue-specific methylated multiplex real-time PCR technique was applied to quantify methylated DNA levels of *DSU* and *SHP1P2* genes in plasma of patients with liver disease (chronic hepatitis B, cirrhosis and HCC) and healthy controls.

Result: The hypermethylation status of *DSU* gene was only found in liver tissues and hepatocyte cell line. Moreover, the normal and tumor liver tissues had significant hypermethylation levels when compared with cholangiocarcinoma ($P<0.001$ and $P<0.05$, respectively). The methylation levels of *DSU* and *SHP1P2* were significantly higher in patients with HCC than healthy controls ($P=0.045$ and $P<0.001$, respectively). Stages and survival rate were associated with higher *SHP1P2* methylation level ($P<0.001$ and $P=0.018$, respectively), but not *DSU* gene.

Conclusion: *SHP1P2* gene is a better epigenetic marker for HCC than *DSU* gene. However, *DSU* gene is a potential forensic marker for criminal investigations because it is a hepatocyte-specific DNA methylation marker.

Keywords: Epigenetic marker, hepatocellular carcinoma, tissue specific DNA methylation, Circulating DNA

บทสรุปผู้บริหาร (Executive Summary) ประกอบด้วย:

- ที่มาและความสำคัญของปัญหา-ทบทวนวรรณกรรม

Increased concentrations of circulating nucleic acids (CNA), as cell-free genomic DNA in plasma and serum, can be detected in numerous human diseases such as different malignancies, myocardial infarction, autoimmune disorders, inflammation, infection, and pregnancy-associated complications. Therefore, the investigation of CNA level can be utilized as a non-invasive, rapid, sensitive and accurate approach for diagnosis in several diseases. Not only the level but also genetic and epigenetic alterations of CNA were detected in patients with cancers for diagnosis and progression. For example, N-ras and K-ras mutations were studied in plasma DNA of myelogenous leukemia and colorectal cancer patients, respectively. Moreover, microsatellite analysis was applied to evaluate loss of heterozygosity (LOH) in circulating DNA of small cell lung carcinoma, head and neck carcinoma, and brain tumor patients. Hypermethylation levels of tumor suppressor genes were observed in different cancers. Several authors also demonstrated that tumor-specific epigenetic markers were the potential biomarkers for discriminating cancer patients from healthy controls and grading disease. Besides tumor-specific epigenetic markers, methylated promoter 2 of *SHP-1* (*SHP1P2*) gene is a tissue-specific methylation marker for epithelial cells and epithelial-derived tumor cells. Vinayanuwattikun et al. quantitated methylation level of SHP1P2 in plasma DNA of advanced non-small cell lung cancer patients. They reported that plasma SHP1P2 methylation could be the potential biomarker for clinical applications in advanced non-small cell lung cancer.

Recently, tissue-specific epigenetic markers have been applied for forensic tissue and biofluid identifications. Forensic DNA methylation analysis has been a novel and alternative method for criminal investigations. Methylation patterns were the outstanding markers for tissue identification because methylation profiles at tissue-specific differentially methylated regions were stable and specific. Furthermore, DNA structure is more stable than RNA structure due to the absence of the 2'-hydroxyl group within every nucleotide of DNA. Therefore, the goal of our pilot study was to explore novel candidates of specific epigenetic markers for several human normal tissue types.

In pilot study, my colleagues and I gathered the methylation microarray databases of all normal human tissues organs from the NCBI website

(http://www.ncbi.nlm.nih.gov/geo/). There were 172 experiments utilized Illumina® HumanMethylation27 BeadChip Kit to detect methylation levels at all 27,578 CpG loci on whole genome. We selected only 39 methylation microarray experiments of 14 types of normal human tissue organs. Subsequently, 39 GEO datasets were used to calculate the mean values of methylation levels in all loci by using CU-DREAM software (Connection up- and down-regulation expression analysis of microarrays). Then, we created 27,578 CpG methylation graphs of each CpG site in R program for comparing the mean value of methylation level among tissue types and discovering tissue-specific CpG methylation locus. The graph of average methylation value of *DSU* gene at cg12620499 site by comparing among 14 normal tissue types. The result indicated hypermethylation at cg12620499 site of *DSU* gene only in liver organ. *DSU* gene was selected to verify the capability of this marker that could be employed as a tissue-specific epigenetic marker for liver tissue. Hence, we used methylation specific PCR (MS-PCR) assay to detect methylation status in different normal tissue types such as kidney, liver, pancreas, adrenal gland, stomach, spleen, and heart. We also designed methylated and unmethylated primers at the region of cg12620499 site in *DSU* gene. From preliminary data, methylation level of *DSU* gene in liver tissue was higher than other tissue types. This result implies that methylation pattern of *DSU* gene could be a promising epigenetic maker for hepatocyte cells and hepatocyte-derived cancer cells. Consequently, serum DNA samples of patients with liver diseases including hepatitis B, hepatitis C, cirrhosis, and hepatoma were measured the methylation level of *DSU* gene by using MSPCR. We observed that methylation levels were significantly higher in cirrhosis and hepatoma patients when comparing with healthy controls ($p = 0.0047$ and $p= 0.0002$, respectively). The data indicated that methylation profile of *DSU* gene might be a new non-invasive marker for diagnosis of liver diseases.

The most common causes of chronic liver disease are a hepatitis B, hepatitis C, alcoholic liver disease, and nonalcoholic steatohepatitis. Chronic injury of liver disease leads to cirrhosis, liver failure, and hepatocellular carcinoma (HCC). Grading liver disease refers to assessing the severity or activity of liver disease. Serum alanine and aspartate aminotransferase tests are the non-invasive tools for identifying liver disease but the levels of these aminotransferases are not always reliable in reflecting disease severity. Liver biopsy has been used to diagnose chronic liver disease and to evaluate grading and staging liver disease. However, it is an invasive test with many possible complications. Therefore, the first aim of this

project is to develop the technique and validate methylation profile of DSU gene as a hepatocyte-specific DNA methylation marker for clinical diagnosis. Real-time probe-based PCR analysis of methylation status technique is used instead of MS-PCR assay because MS-PCR method is the manual methylation scoring based on interpretation of gel band intensities and low-throughput tool. In second aim, we detect the methylation level of DSU gene in plasma and serum DNA of patients with liver disease to determine whether this epigenetic maker can be applied as a non-invasive test for diagnosis, evaluation (e.g. grade or stage) and monitoring progression of liver disease.

- วัตถุประสงค์

The main objectives of this study are

1. To develop the technique and validate methylation profile of *DSU* gene as a hepatocyte-specific DNA methylation marker for clinical diagnosis.

2. To determine whether *DSU* and *SHP1P2* genes can be applied as epigenetic markers for liver diseases.

- วิธีทดลอง

1. To confirm and identify the methylation patterns at individual CpG sites within the defined region of *DSU* gene by using pyrosequencing

Pyrosequencing is the sequencing by synthesis technique. This method can be applied to quantify DNA methylation at specific CpG sites within the target region of interest. Before pyrosequencing for DNA methylation quantification can occur it is essential to bisulfite-convert the DNA sample. The results of bisulfite pyrosequencing are displayed as a pyrogram with percent methylation values for each site. Hence, the methylation levels at individual CpG sites were compared among different normal tissue types, paraffin-embedded hepatocellular carcinoma and cholangiocarcinoma tissues, and cell lines to confirm that this marker was hypermethylated in hepatocyte tissue and cell but not in other tissue and cell types.

Normal tissue types

Paraffin embedded tissues of kidney, liver, pancreas, adrenal gland, stomach, spleen, and heart from autopsy samples.

Abnormal tissue types

paraffin-embedded hepatocellular carcinoma and cholangiocarcinoma tissues were obtained from Department of pathology at Chulalongkorn university.

Cell lines

Cell lines	Derivation	Cell type
Jurkat	T-cell leukemia	Lymphocyte
Daudi	Burkitt's lymphoma	B lymphoblast
Huh7	Hepatocellular carcinoma	Hepatocyte
HepG2	Hepatocellular carcinoma	Hepatoblast
Hela	Cervical cancer	Epithelium

2. To validate the technique for the quantification of DNA methylation biomarkers

Real-time probe-based PCR analysis of methylation status technique was used to quantify methylation level instead of MS-PCR. This method is standardized and high-throughput method. From pyrosequencing result, we obtained evidence that confirm the pattern of methylation across the specific domain of *DSU* gene. Therefore, primer set and probe was designed to match the methylated sequences at this region. It may affect to sensitivity and specificity of this marker.

2.1 Verification of DSU methylation marker as a hepatocyte-specific marker

We mixed DNA samples of three different cell lines in various proportions of each cell line. Combined cell line samples were generated to test sensitivity and specificity of real-time probe-based PCR assay by using DSU methylation marker. Each experiment included several non-template controls (water) and an unmethylated control (bisulfite treated leukocyte DNA; Daudi cell line). A standard curve was created for each experiment using a serial dilution of the methylated control (bisulfite converted hepatocyte DNA; Huh7 cell line).

2.2 Development of tissue-specific methylated multiplex real-time quantitative PCR as a powerful method for diagnosing liver disease

As mentioned above, CNA of healthy people derives from apoptosis of lymphocytes and other nucleated cells. Also, CNA of patients with diseases contain additional cellular sources of circulating DNA. Therefore *DSU* and SHP1P2 loci were utilized to identify methylation pattern in hepatocytes and epithelial cells respectively. Epithelial tissues line the cavities and surfaces of structures throughout the body. Hepatocytes are epithelial cells of liver organ. The methylation of SHP1P2 is ubiquitous in all epithelial cells. We expected that methylated multiplex real-time quantitative PCR might be a powerful quantitative technique for diagnosing liver disease. We designed specific three sets of both primers and probes. In the first step, we detected methylation profiles in mixed DNA samples of three cell lines by

using a multiplex methylation-specific PCR for optimizing condition. Next step, a multiplex methylation-specific PCR was utilized to evaluate methylation patterns in plasma DNA of patients with liver disease and healthy controls. Final step, we studied the relationship between clinical and pathological characteristics of the liver disease (e.g., stage or grade) and methylation level of each methylation maker.

- แผนการดำเนินงาน

Activities in the first year	Months											
	1	2	3	4	5	6	7	8	9	10	11	12
1. Identification of the methylation patterns in different normal tissues and cell lines by pyrosequencing	←	→										
2. Verification of DSU methylation marker			←	→								
3. Conditional optimization of Multiplex real-time quantitative PCR					←	→	→					
4. Evaluation of methylation patterns in plasma DNA of patients with liver disease by Multiplex real-time quantitative PCR								←	→	→	→	→

Activities in the second year	Months											
	1	2	3	4	5	6	7	8	9	10	11	12
5. Evaluation of methylation patterns in plasma DNA of patients with liver disease by Multiplex real-time quantitative PCR (continue)	←	→	→	→	→	→						
6. Data analysis							←	→	→			
7. Summary and report										←	→	→

ผลการทดลอง

1. To confirm and identify the methylation patterns at individual CpG sites within the defined region of *DSU* gene by using pyrosequencing

The results of bisulfite pyrosequencing were displayed as the pyrograms with percent methylation value of each CpG sites. For instance, **Figure 1** illustrated the partial pyrograms of Daudi, HepG2, Huh7 and a normal liver tissue. The percent methylation level of individual CpG sites among five cell lines and nine normal tissue types were plotted and represented in **Figure 2** and **Figure 3**, respectively. The paraffin embedded tissues of normal liver, HCC and cholangiocarcinoma were quantified the methylation level at specific CpG sites of *DSU* gene by using pyrosequencing. We found both normal liver tissues and HCC had significant hypermethylation levels when compared with cholangiocarcinoma **(Figure 4)**. These findings suggested that *DSU* gene was a hepatocyte-specific methylation marker. Next investigation, we developed and validated the technique to detect this epigenetic marker for clinical applications in liver disease.

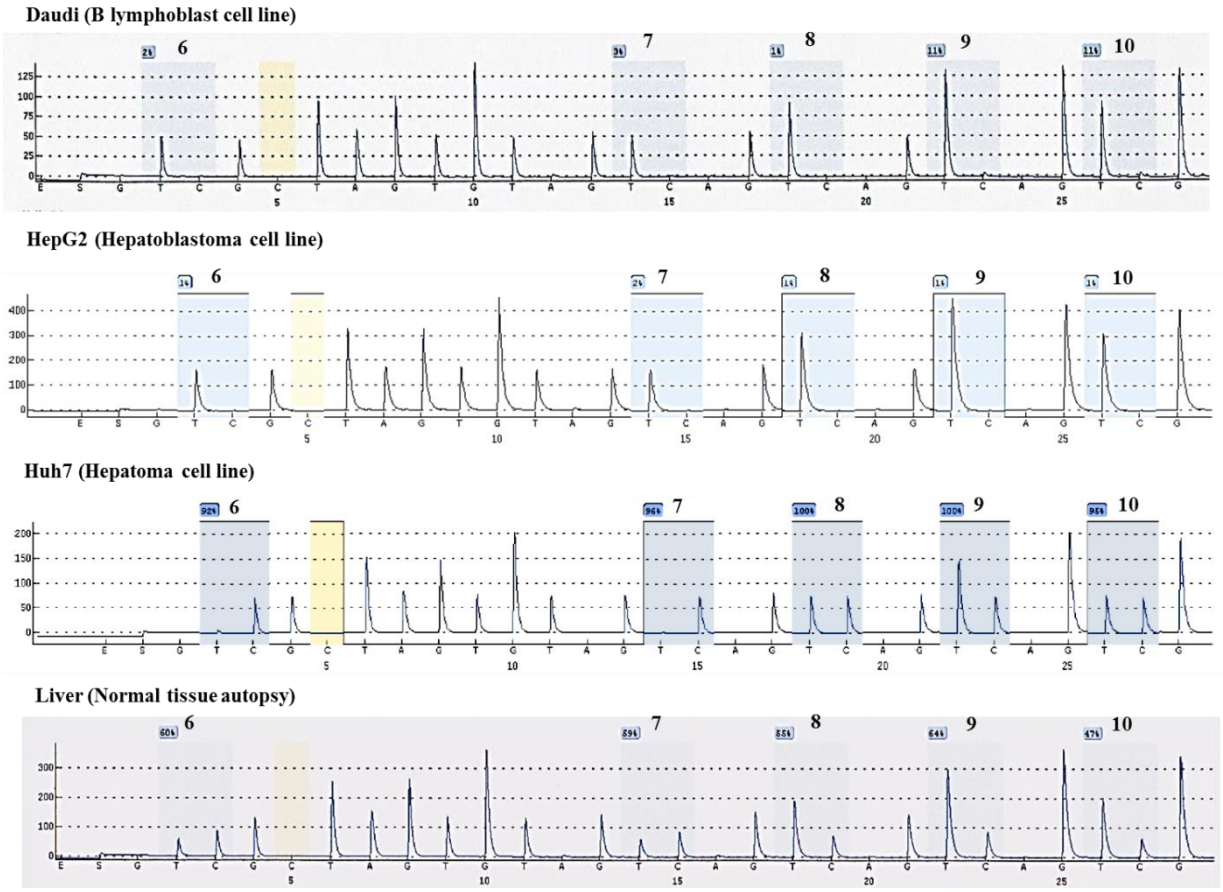

Figure 1. The partial pyrograms demonstrate bisulfite pyrosequencing results of *DSU2* pyrosequencing primer. In each CpG site, the C-peak or T-peak represents the methylated and unmethylated sequence respectively.

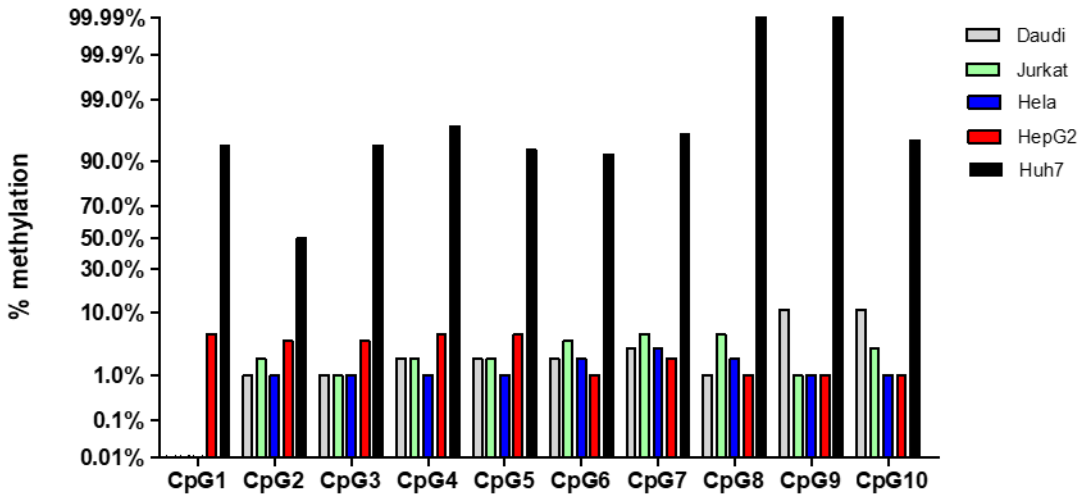

Figure 2. Quantification of percent methylation levels in each CpG site of the *DSU* gene in different cell lines.

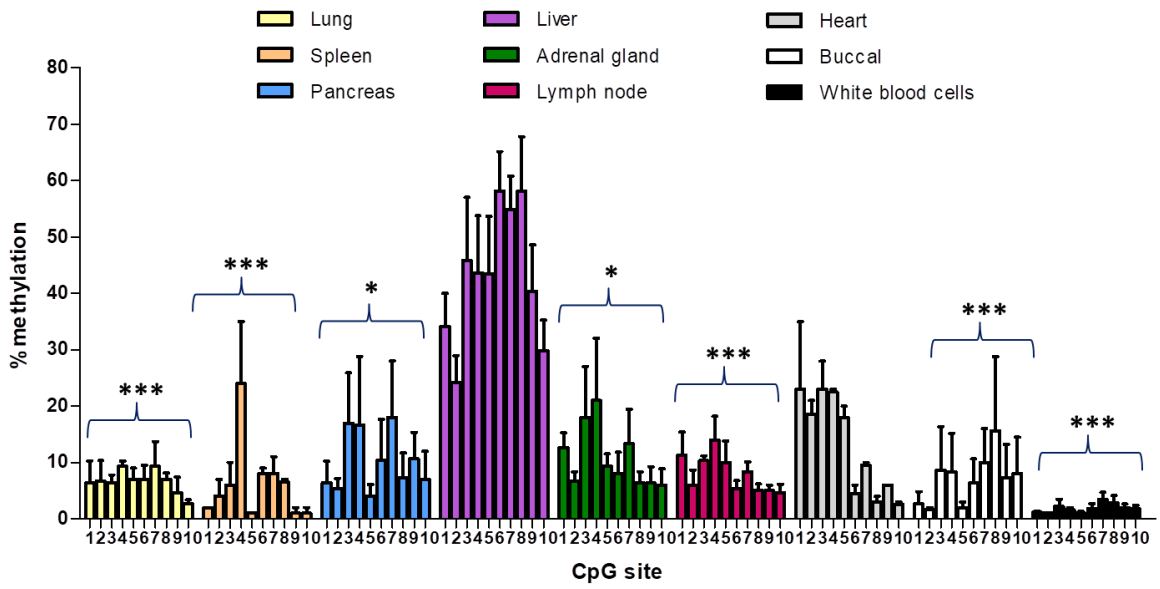

Figure 3. Quantification of percent methylation in each CpG site of the *DSU* gene in different normal tissue types and comparison of average percent methylation levels between liver and each normal tissue type. One asterisk indicates a p-value lower than 0.05, and three asterisks indicate p-value lower than 0.001.

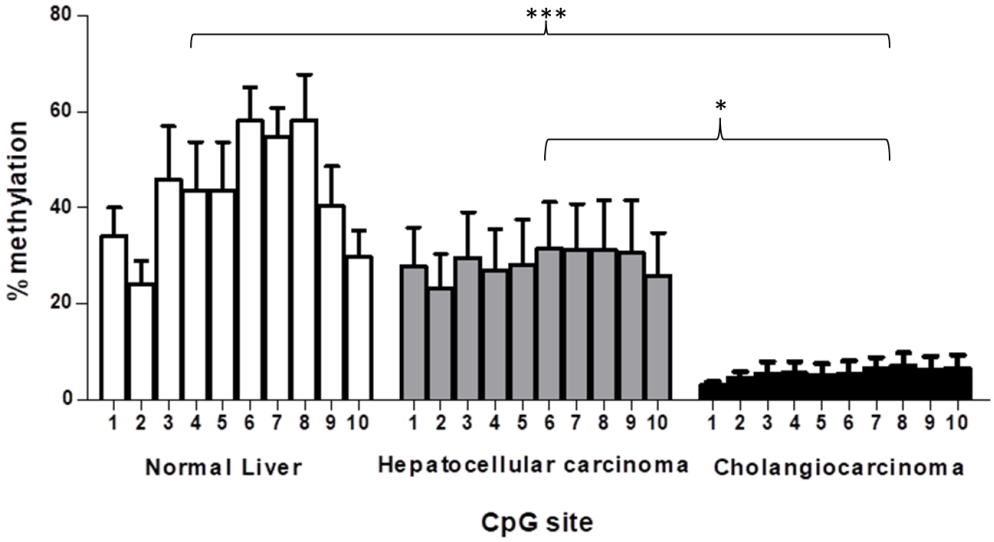

Figure 4. Quantification of percent methylation levels in each CpG site of the *DSU* gene and Comparison of average percent methylation levels among normal liver (tissue autopsy), hepatocellular carcinoma (Tissue biopsy) and Cholangiocarcinoma (Tissue biopsy). One asterisk indicates a p-value lower than 0.05, and three asterisks indicate p-value lower than 0.001.

2. To validate the technique for the quantification of DNA methylation biomarkers

2.1 Verification of *DSU* methylation marker as a hepatocyte-specific marker

We combined among three primer sets and probes of these genes to quantify methylated DNA levels of *DSU* and *SHP1P2* genes, which is termed “tissue-specific methylated multiplex real-time PCR technique”. In the first step, we optimized condition and validated this technique by detecting methylation profiles in mixed bisulfite treated DNA samples of Huh7, HepG2 and Daudi cell lines. For *DSU* gene, the positive control was bisulfite treated DNA sample of Huh7 and the negative controls were bisulfite treated DNA samples of HepG2 and Daudi. Hepatocytes are the epithelial cells of the liver; therefore, bisulfite treated DNA samples of Huh7 and HepG2 were the positive controls for *SHP1P2* gene. Bisulfite treated DNA sample of Daudi was the negative control for this gene because of a hematopoietic cell. We prepared the same DNA concentration of three cell lines before performing the PCR. **Figures 5A 5B** and **5C** manifested the ΔRN values of the Beta actin, *DSU* and *SHP1P2* probe signals, respectively. The validation results showed that this technique and these epigenetic markers would be potential method and epigenetic markers for plasma DNA detection in clinical applications of liver disease.

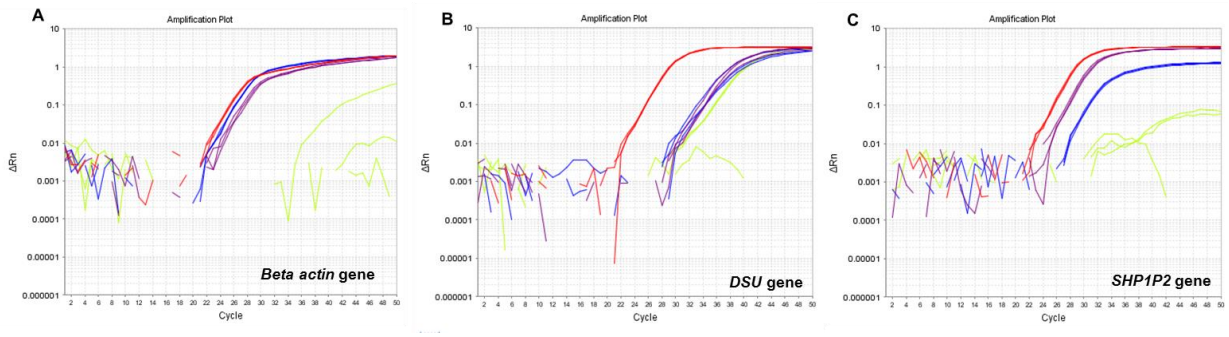

Figure 5. Validation of tissue-specific methylated multiplex real-time PCR technique. (A), (B) and (C) show the ΔRN values of Beta actin, *DSU* and *SHP1P2* probe signals, respectively. Red, Blue and purple represent bisulfite treated DNA samples of Huh7, Daudi and HepG2, respectively. Green is non-template control (water).

2.2 Development of tissue-specific methylated multiplex real-time quantitative PCR as a powerful method for diagnosing liver disease

To determine the methylation levels of *DSU* and *SHP1P2* in blood sample from patients with CHB, HBV-HCC and healthy controls. Methylated DNA was performed by using real-time PCR with Methylation-Specific primer TaqMan probed. **Figure 6A** indicated that the mean ΔRN value of *DSU* of the healthy controls, patients with CHB and HBV-HCC showed 0.13±0.23, 0.22±0.37 and 0.22±0.37, respectively. The mean ΔRN of SHP1P2 were 0.06±0.06 for the healthy controls, 0.05±0.05 for CHB and 0.18±0.15 for the HBV-HCC group (**Figure 6B**). To explore whether there was a significant methylation level change of *DSU* and *SHP1P2* in different BCLC stages of HCC. We compared the methylation level in methylated DNA between early stage (0-A) and later stage (B-C). **Figure 7A** demonstrated that the methylation level of *SHP1P2* was significantly increased in patients with B-C stage compared with in 0-A stage (0.22±0.17 vs. 0.11±0.08, P<0.001). However, there was no significantly difference between 0-A and B-C stages in *DSU* methylation level (0.27±0.41 vs. 0.23±0.38, P=0.619) (**Figure 7B**). The methylation levels of *DSU* and *SHP1P2* were compared with receiver operating characteristic (ROC) curves. **Figure 8** showed that the area under the curve (AUC) is 0.600 for *DSU* (95%CI: 0.53 to 0.67, P=0.004) and 0.794 for *SHP1P2* (95%CI: 0.74 to 0.85, P<0.001). The sensitivity and specificity of *DSU* were 72.3% and 45.5% respectively; whereas, the sensitivity and specificity of *SHP1P2* were 61.2% and 84.3% respectively. The patients were divided into two groups based on the cut-off value of *DSU* and *SHP1P2* methylation levels. Accordingly, there were patients with *DSU* and *SHP1P2* as low (<0.1) and high (≥0.1) of methylation levels groups. The

median overall survival (OS) of patients with low and high *DSU* levels were 29 and 27 months (P=0.891 by log rank test) (**Figure 9**). Interestingly, patients with low *SHP1P2* methylation level had the median OS at 43 months, which was significantly better than that of patients whose high *SHP1P2* level (median OS, 25 months; P=0.018).

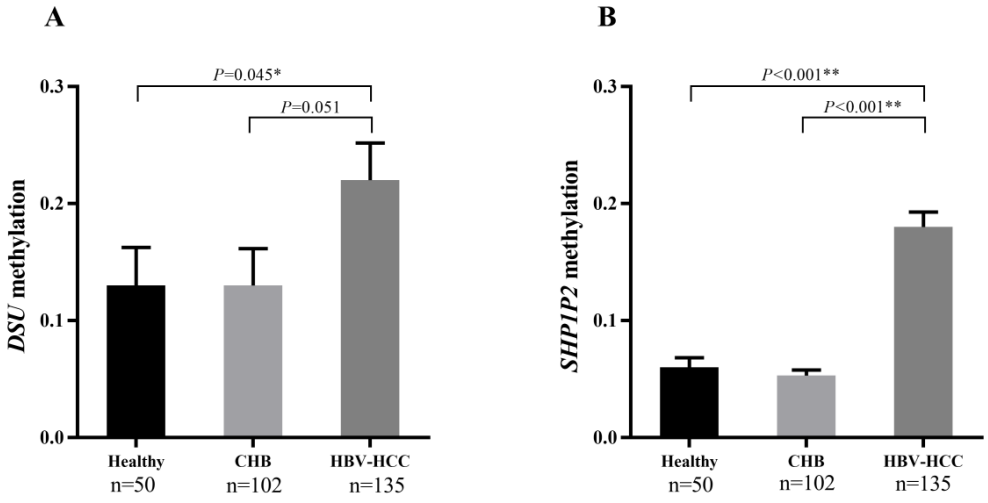

Figure 6. Comparison plasma methylation levels among studied group (A) *DSU* methylation level (B) *SHP1P2* methylation level.

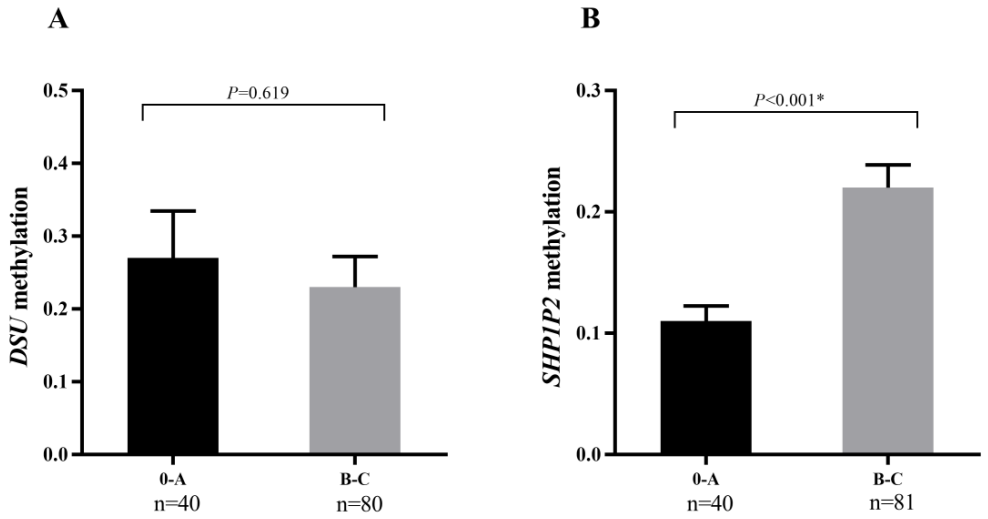

Figure 7. Plasma methylation levels regarding to BCLC stage in patients with HCC (A) *DSU* methylation level (B) *SHP1P2* methylation level.

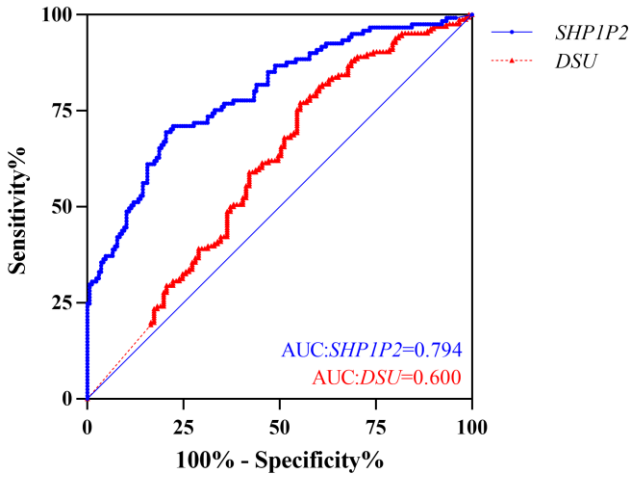

Figure 8. Receiver operating characteristic curves of DSU and SHP1P2 methylation levels.

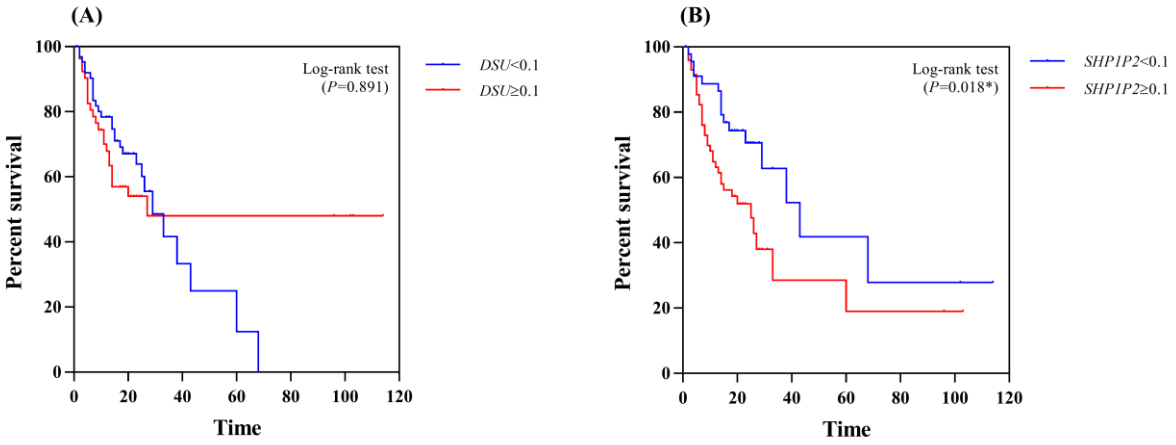

Figure 9. Overall survivals of patients with HCC regarding to methylation levels (A) *DSU* methylation level (B) *SHP1P2* methylation level.

สรุปและวิจารณ์ผลการทดลอง และข้อเสนอแนะสำหรับงานวิจัยในอนาคต

Although the methylated CpG site of *DSU* gene is not a potential biomarker for clinical diagnosis of hepatocellular carcinoma (HCC), this marker is a hepatocyte-specific DNA methylation marker. It is able to be a forensic marker for identification of organ tissue type from crime scene because the methylated sequence of DSU gene is a specific organ marker but not a specific tumor marker. On the other hand, SHP1P2 gene is an efficient biomarker for clinical applications in HCC. This marker is an epithelial-specific DNA methylation marker. Thus, this marker is possible to be a biomarker for identifying the original tissue type but not identifying the specific organ. It is appropriate for use in clinical applications of carcinomas.

ภาคผนวก (ขอเป็นเอกสารปกปิด)

ฉบับร่างของผลงานตีพิมพ์ คาดว่าจะตีพิมพ์ในวารสารวิชาการนานาชาติ

Hepatocyte-specific methylation markers in circulating nucleic acids: Potential non-invasive tool for clinical diagnosis of liver disease

Abstract

Background: Epigenetic alterations of circulating DNA are detected in patients for diagnosis and progression of diseases. *SHP1P2* genes was utilized to be an epithelial-specific DNA methylation marker. For a hepatocyte-specific DNA methylation marker, we was validated methylation profile of *DSU* gene. Then, we investigated whether *DSU* and *SHP1P2* genes could be non-invasive epigenetic markers for liver diseases.

Method: Pyrosequencing was used to identify the methylation patterns of *DSU* gene by comparing the methylation levels among different normal tissue types, different cell lines and tissue samples of HCC and cholangiocarcinoma. Tissue-specific methylated multiplex real-time PCR technique was applied to quantify methylated DNA levels of *DSU* and *SHP1P2* genes in plasma of patients with liver disease (chronic hepatitis B, cirrhosis and HCC) and healthy controls.

Result: The hypermethylation status of *DSU* gene was only found in liver tissues and hepatocyte cell line. Moreover, the normal and tumor liver tissues had significant hypermethylation levels when compared with cholangiocarcinoma ($P<0.001$ and $P<0.05$, respectively). The methylation levels of *DSU* and *SHP1P2* were significantly higher in patients with HCC than healthy controls ($P=0.045$ and $P<0.001$, respectively). Stages and survival rate were associated with higher *SHP1P2* methylation level ($P<0.001$ and $P=0.018$, respectively), but not *DSU* gene.

Conclusion: *SHP1P2* gene is a better epigenetic marker for HCC than *DSU* gene. However, *DSU* gene is a potential forensic marker for criminal investigations because it is a hepatocyte-specific DNA methylation marker.

Introduction

Circulating nucleic acid (CNA) denotes the presence of cell-free DNA and RNA in the bloodstream and other body fluids (1). The cell-free nucleic acids are derived from dead cells via apoptosis and necrosis as passive mechanisms (2-5). Moreover, cells can spontaneously release nucleic acids into the blood as active mechanisms (6). CNA can be detected in both healthy subjects and patients with nonmalignant and malignant diseases (7, 8). However, healthy people have low levels of plasma DNA, which is originated from apoptosis of leukocytes and other nucleated cells (9). The occurrence of elevated CNA levels has been found in patients with malignant diseases such as colon (10, 11), pancreas (11), prostate (12), breast (10, 13), lung (10, 14), and liver (15) cancers. Furthermore, the correlation between increased plasma DNA levels and tissue damage has been reported in patients with acute stroke, acute trauma, and myocardial infarction (6). The physiological and pathological processes are the causes of increased concentrations of CNA in patients (9). Therefore, the investigation of CNA level has been utilized as a non-invasive, rapid, sensitive and accurate approach for screening, prognosis, and diagnosis in several diseases. Not only the level but also genetic and epigenetic alterations of CNA were detected in patients for diagnosis and progression of malignant diseases. For example, *N-ras* and *K-ras* mutations were studied in plasma DNA of myelogenous leukemia (16) and colorectal cancer patients (17), respectively. Moreover, microsatellite analysis was applied to evaluate loss of heterozygosity (LOH) in circulating DNA of

small cell lung carcinoma (18), head and neck carcinoma (19), and brain tumor patients (20). Hypermethylation levels of tumor suppressor genes were observed in various cancers. Several authors also demonstrated that tumor-specific epigenetic markers were the potential biomarkers for discriminating cancer patients from healthy controls and grading diseases (20, 21). Besides tumor-specific epigenetic markers, methylated promoter 2 of *SHP-1* (*SHP1P2*) gene is a tissue-specific methylation marker for epithelial cell lines and epithelial tissues of several organs including lung, liver, breast, kidney and skin (22). Consequently, this marker was applied to be a diagnostic, prognostic and lymph node metastatic markers of lung and colorectal cancers. There were found high methylation level of *SHP1P2* in plasma and lymph node of lung cancer patients (23, 24). In addition, the quantity of *SHP1P2* methylation in lymph nodes of colorectal cancer patients was higher than in lymph nodes of controls (25). The recent study has documented that tissue-specific methylation markers can also be used to identify cell types of circulating DNA in human because dead cells release the fragments of tissue-specific DNA methylation into bloodstream such as unmethylated CpG sites at *MBP3* locus as an epigenetic marker of oligodendrocytes (26). Additionally, tissue-specific epigenetic markers have been applied for forensic tissue (27) and biofluid identifications (28). Forensic DNA methylation analysis has been a novel and alternative method for criminal investigations. Methylation patterns were the outstanding markers for tissue

identification because methylation profiles at tissue-specific differentially methylated regions were stable and specific (29).

Hepatocellular carcinoma (HCC) is the sixth most frequently occurring malignancy in the world and is the second most common cause of global cancer mortality. Asia and Africa are the regions with the highest incidence rates of liver cancer (30). Chronic liver disease can develop into HCC. The etiology of chronic liver disease includes viral infection (hepatitis B and C), alcohol, metabolic diseases and aflatoxin. Ultrasonography, computed tomography (CT) and magnetic resonance tomography (MRI), along with alpha-fetoprotein (AFP) measurement have been recommended for periodic screening in patients with chronic hepatitis and for diagnosis of HCC. Noninvasive imaging technologies have low sensitive for tumor size less than 1 cm (31). The level of AFP is not reliable in reflecting on the severity of disease, as some patients with HCC cannot produce high levels of AFP. Liver biopsy is considered for diagnosis of HCC and evaluation of cancer staging. However, the biopsy is an invasive assay with many possible complications (32). Currently, circulating DNA is recognized as non-invasive method and is used for clinical diagnosis in many human diseases, as well as for HCC. It has been reported that the plasma DNA levels in patients with HCC were higher than patients with HBV-related liver fibrosis; nevertheless, the increased concentration of plasma DNA was not associated with stage of HCC development. Thus, quantitative analysis of CNA is not sufficient for early detection, diagnosis, and staging assessment in HCC. Early

detection plays an important role in improving the effectiveness of cancer treatment. Additionally, quantitative analysis of CNA is not specific for liver cancer because elevated plasma DNA levels are found in many diseases. Therefore, non-invasive and potential biomarker is essential for clinical applications in HCC.

From the previous study, our colleagues analyzed methylation microarray data to identify tissue-specific methylation by using bioinformatics approach (33). The 34 single organ hypermethylated CpG loci and 52 hypomethylated CpG loci were discovered. The CpG at 12620499 position of *DSU* gene was the one of 34 single organ hypermethylated CpG loci. This cg12620499 locus demonstrated high methylation level in only liver organ. As mentioned above, *SHP1P2* gene is a tissue-specific methylation marker for epithelial tissues of many organs including liver organ. For increasing sensitivity and specificity of CNA analysis in patients with HCC, *DSU* and *SHP1P2* genes were utilized to be hepatocyte-specific and epithelial-specific DNA methylation markers, respectively. The first objective of this study was to identify and validate methylation profile of *DSU* gene as a hepatocyte-specific DNA methylation marker. The second objective was to develop the technique and investigate whether *DSU* and *SHP1P2* genes could be non-invasive epigenetic biomarkers for early detection, diagnosis, staging assessment and monitoring progression of HCC. Thus, tissue-specific methylated multiplex real-time PCR technique was applied to quantify methylated DNA levels of *DSU* and *SHP1P2* genes in plasma of patients with liver disease and healthy controls.

Materials and Methods

Ethics Statement

The study protocol was reviewed and approved by the institutional review board of Faculty of Medicine, Chulalongkorn University in Bangkok, Thailand (IRB 564/57). All subjects gave written informed consent.

Subject population and Specimen collection

To verify whether *DSU* gene could be a hepatocyte-specific DNA methylation marker, we compared the methylation levels at individual CpG sites among different normal tissue types, different cell lines and tissue samples of hepatocellular carcinoma and cholangiocarcinoma. Pyrosequencing is the sequencing by synthesis technique. This technique was applied to quantify DNA methylation level at individual CpG sites within the defined region, which covered Cg12620499 locus of *DSU* gene (33). For normal tissue types, paraffin embedded tissues of several organs including lung, pancreas, heart, spleen, adrenal gland and lymph node were obtained from three autopsy cases, except for liver tissues, which were collected from eight autopsy cases. All of tissue samples were approved of microscopic examination. Buccal cells, which were normal epithelial cells, were collected from three healthy controls by rinsing the mouth with 10 mL of normal saline. Normal white blood cells were obtained from the same healthy controls by using EDTA blood tubes. The cell lines used were Jurkat (T lymphocyte; derived from T-cell leukemia), Daudi (B lymphoblast; derived from Burkitt's lymphoma), Hela (Epithelial cell; derived from Cervical cancer), HepG2

(Hepatoblast; derived from Hepatocellular carcinoma) and Huh7 (Hepatocyte; derived from Hepatocellular carcinoma). Most of cell lines were purchased from the ATCC, except for Huh 7, which was kindly acquired from Associate Professor Sunchai Payungporn, Department of Biochemistry, Faculty of Medicine, Chulalongkorn University. Hematopoietic and epithelial cell lines were cultured in RPMI1640 and Dulbecco's Modified Eagle's medium (Gibco, Waltham, USA), respectively. Huh7 was grown in Eagle's minimal essential medium (Gibco, Waltham, USA). Each of culture media was supplemented with 10% fetal bovine serum and 1% antibiotic-antimycotic agent (Gibco, Waltham, USA). The cell lines were incubated at 37 ℃ in a humidified atmosphere and 5% CO_2. For tumor tissues, paraffin embedded biopsies were retrieved from the Department of Pathology, Chulalongkorn Memorial Hospital between 2012 and 2014. Twelve hepatocellular carcinomas and twelve cholangiocarcinomas were diagnosed by pathologists and included in this experiment. One of the co-author, as a pathologist, reviewed histological slides again to identify the lesions of malignant cells. Then, all samples were microdissected to isolate cancer tissues from their adjacent normal tissues. To investigate tissue-specific methylated multiplex real-time PCR technique as a powerful method for liver disease, *DSU* and *SHP1P2* genes were utilized to be the tissue-specific methylation markers for hepatocytes and epithelial cells, respectively. Tissue-specific methylated multiplex real-time PCR technique was manipulated to evaluate methylated DNA levels in plasma of patients with liver disease and healthy controls. Plasma samples used in this

project were obtained from 50 healthy controls and 223 patients with chronic liver disease (57 patients with chronic hepatitis B (CHB), 45 patients with cirrhosis (CHB-cirrhosis) and 121 patients with HCC), who attended at King Chulalongkorn Memorial Hospital, Bangkok. All blood samples were collected and stored at -80^0C until tested. All subjects gave written informed consent for the study and the protocol was approved by the Ethics Committee, Faculty of Medicine, Chulalongkorn University. The diagnosis of chronic hepatitis was based on the presence of prolonged elevation of serum ALT and/or histologically proven. The diagnosis of cirrhosis was based on histopathology and/or clinical features (e.g. the presence of spider nevi and palmar erythema, radiological findings (e.g. architectural changes in the liver typical of cirrhosis and splenomegaly) and/or upper endoscopic findings (e.g. the presence of esophageal or gastric varices). The diagnosis of HCC was based on typical imaging studies and/or histopathology (fine needle aspiration, core liver biopsy or surgical resection) Diagnostic criteria of HCC by imaging modalities were based on reports of focal lesions with hyper-attenuation at the arterial phase, hypo-attenuation at the portal phase in dynamic CT or MRI. In cases without typical imaging features, liver biopsy was performed to confirm the diagnosis of HCC.

DNA preparation and Bisulfite treatment

Before Paraffin embedded tissues were microdissected for cell collection, they were deparaffinized with a xylene-based protocol. Then, a standard phenol/ chloroform technique was utilized to extract DNA from the cells. For circulating

DNA, the same method was used to extract DNA from 100 – 200 ul of plasma samples. The DNA materials were converted to bisulfite DNA by using EZ DNA Methylation-Gold^{TM} Kit (Zymo research, Orange, USA) and following the manufacturer's instructions.

Pyrosequencing

PCR and pyrosequencing primers were designed by using Methprimer program (http://www.urogene.org/cgi-bin/methprimer2/MethPrimer.cgi). The primer-binding regions covered the Cg12620499 locus of *DSU* gene (33), which interrogated and quantified the methylated fraction (%) in the CpG sites. PCR was performed by using Mastercycle® pro (Eppendorf, Hamburg, Germany) and PyroMark PCR kit (Qiagen Inc.). Each PCR reaction had volume 50 µL containing 1x PyroMark PCR master mix, 0.2 µM of forward (5'GGGGTTGAGGGATTGGTTGAG3') and reverse (5' Biotin-ACTACRACCCAAATAAACCCAAAT3') primers, 1x CoralLoad concentrate and 2 µL of bisulfite DNA. The PCR products were amplified using initial denaturation at 95^{O}C for 10 minutes followed by 45 cycles of 95^{O}C 30 seconds, 60^{O}C for 1 minute, and 72^{O}C for 1 minute and then final extension at 72^{O}C for 5 minutes. Five microliters of PCR products were visualized using 8% polyacrylamide gel electrophoresis stained with SYBR DNA gel stain (Invitrogen Life technologies, Carlsbad, CA, USA) to detect the band of PCR product. Subsequently, The PCR products were purified and denatured according to manufacturer's instruction (Qiagen Inc.). Then the single-stranded PCR products were annealed to 0.3 µM

pyrosequencing primers as detailed in **Table 1** and pyrosequencing was performed by using PyroMark Q96ID (Qiagen Inc.). The nucleotide dispensation orders and sequence to analyze were shown in **Table 1**.

Tissue-specific methylated multiplex real-time quantitative PCR

Tissue-specific methylated multiplex real-time PCR technique was manipulated to evaluate methylated DNA levels in plasma of patients with liver disease and healthy controls. We modified Combined Methylation-Specific primer TaqMan real-time (COMST) PCR technique (25) to quantitate methylated DNA level of *DSU* gene. Therefore, only methylated set of primers and probe of this gene was designed. The oligonucleotide sequences of forward primer, reverse primer and probe were 5'GGGGTTGAGGGATTGGTTGAG3', 5'ACTACGACCCAAATAAACCCAAAT3' and 5'VIC-CACCTAACGAAAAACTC-MGB3', respectively. For *Beta actin* and *SHP1P2* genes, we applied the sets of primers and probes as established by Rattanatanyong *et al* (25). The multiplex real-time quantitative PCR were used to quantitate the methylated DSU, methylated SHP1-P2 and beta actin sequences. Real-time PCR were performed by using 1x TaqMan GTXpress real time PCR master mix (ABI, USA) with 0.7 µM of DSU primers, 0.9 µM of Beta actin and SHP1P2 primers and 2 nM of each probes, operated by an ABI 7500 fast real-time PCR system. The standard curve of *DSU* and *SHP1-P2* genes were generated by bisulfite treated DNA sample of

Huh7. For calculation, the copy number of methylated DSU and methylated SHP1-P2 were normalized by copy number of beta actin.

Statistical analysis

All statistical analyses were performed using the Statistical Program for Social Sciences (SPSS for Windows, SPSS Inc., Chicago, IL) version 22. Student t test and Mann–Whitney U test were used to test continuous variables with normal and skewed distribution, respectively. Correlation analyses were performed after logarithmic transformation of data with skewed distributions and tested by Pearson's correlation analysis. Categorical variables were tested by Chi-square test or Fisher's exact test. Survival curves were analyzed using the Kaplan-Meier method and differences between curves were assessed by the log-rank test. The Cox regression analysis was performed to identify independent factors associated with overall survival (OS) of patients with HCC. Statistical significance is defined by a *P* value of less than 0.05.

Table 1. Pyrosequencing primers, dispensation orders and sequences to analyze

Pyrosequencing primer		Dispensation order (Sequence to analyze)
DSU1	5'GAGGGATTGGTTGAGAAT3'	GTCGTGTGCTGTGTAGTCGTAGTCGAGTGTGAGATGTCAGTC (YGTGTGTTGTTGTTGYGGGTGYGAGTGTTTGGAGGAGYGYG)
DSU2	5'GTTTGAGAAGGAGTTTTT3'	GTCGCTAGTGTAGTCAGTCAGTCAGTCG (YGTTAGGTGGGTGYGTYGTTYGGGTYGGGA)

Y means C or T

Results

DSU methylation status as a hepatocyte-specific DNA methylation marker

As mentioned previously, our colleagues discovered that the Cg12620499 locus of *DSU* gene demonstrated high methylation level in only liver tissue by bioinformatics analysis (33). Consequently, we hypothesized that methylation status of *DSU* gene could be a tissue-specific epigenetic marker for liver organ. The first aim of this experiment was to identify the methylation patterns at individual CpG sites within the defined region of *DSU* gene by using pyrosequencing and to compare the percent methylation levels of each CpG sites among different cell lines and normal tissue types. We designed one set of PCR primers and two pyrosequencing primers, which covered the CpG at position 12620499 of *DSU* gene. The cg12620499 has been located in the region of CpG islands; thus, ten CpG sites within the target region of this gene were investigated (**Figure 1A**). The position 5 of ten CpG sites was the CpG at position 12620499 of *DSU* gene. The DSU1 and DSU2 pyrosequencing primers were used to quantitate the percent methylation value at positions 1 to 5 and 6 to 10 of CpG sites, respectively (**Table 1**). The results of bisulfite pyrosequencing were displayed as the pyrograms with percent methylation value of each CpG sites. For instance, **Figure 1B** illustrated the partial pyrograms of Daudi, HepG2, Huh7 and a normal liver tissue. The percent methylation level of individual CpG sites among five cell lines and nine normal tissue types were plotted and represented in **Figure 2** and **Figure 3**, respectively. We predicted that Huh7 and HepG2 should have high

percentage of methylation level at all CpG sites of *DSU* gene because they were derived from liver cancer, whereas Daudi and Jarkat as hematopoietic cell lines and Hela as a cervical cancer cell line should have low percentage of methylation level at all CpG sites of this gene. Unexpectedly, we found that only Huh7 had hypermethylation level but HepG2 had hypomethylation level of *DSU* gene, as well as other cell lines (**Figure 2**). Even though Huh7 and HepG2 were originally obtained from liver tissue, the pyrogram of HepG2 was different from Huh7 and a normal liver tissue (**Figure 1B**). Form the previous report, Huh7 was established from hepatocellular carcinoma of a 57-year old Japanese male; while HepG2 was established from hepatoblastoma of a 15-year old Argentinean male (34). Hepatoblastoma is also an embryonic malignancy of hepatocellular origin (35). These investigations showed that Huh7 was a mature hepatocyte and HepG2 was an immature hepatocyte; therefore, the methylation pattern of *DSU* gene in HepG2 was distinct from Huh7. Moreover, **figure 3** demonstrated that the methylation levels of all ten GpC sites in different normal tissue types. The comparison of average percent methylation values between normal liver tissue and each other tissue types were manifested that the average percent methylation levels in liver tissue were significantly higher than in lung tissue, spleen tissue, pancreas tissue, lymph node, adrenal gland, buccal tissue and white blood cell. No significant difference was found in the heart tissue although all ten CpG sites of *DSU* gene in the liver tissue had higher methylation values than heart tissue. Then, we compared the percent

methylation levels in each CpG sites between normal liver tissue and each other tissue types (**Table 2**). Heart tissue was discovered that the methylation levels at positions 1 to 5 of CpG sites were insignificant difference; conversely, the methylation levels at positions 6 to 10 of CpG sites were significant difference when compared with liver tissue. For this reason, the mean of percent methylation values did not differ significantly between liver and heart tissues. The DNA methylation values at positions six to nine of CpG sites in liver tissue were significant higher than in other normal tissue types, whereas the DNA methylation at position 2 of CpG sites was no statistically significant difference between liver tissue and other normal tissue types. Base on the data of pyrosequencing, the hypermethylation status of *DSU* gene was found in normal liver tissue and Huh7. These observations indicated that *DSU* gene could be a hepatocyte-specific DNA methylation marker for adult liver cells. Besides hepatocytes, bile duct epithelial cells are the component of histological structure of liver tissue. For this reason, the second aim was to confirm that this gene was a hepatocyte-specific methylation marker and could be applied for clinical diagnosis in liver disease. The paraffin embedded tissues of normal liver, HCC and cholangiocarcinoma were quantified the methylation level at specific CpG sites of *DSU* gene by using pyrosequencing. We found both normal liver tissues and HCC had significant hypermethylation levels when compared with cholangiocarcinoma ($P<0.001$ and $P<0.05$, respectively) (**Figure 4**). It was also found that the mean values of percent methylation between the liver and HCC were not significantly different.

These findings suggested that *DSU* gene was a hepatocyte-specific methylation marker. Next investigation, we developed and validated the technique to detect this epigenetic marker for clinical applications in liver disease.

Development and validation of tissue-specific methylated multiplex real-time PCR technique

Real-time probe-based PCR analysis was applied to quantify methylated DNA levels because this technique was standardized, sensitive, specific and high-throughput. Epigenetics of *DSU* and *SHP1P2* genes were utilized to be the specific markers for identifying hepatocytes and epithelial cells, respectively. As described in materials and methods, the methylated primers set and probe of *DSU* gene were designed for analyzing only methylated sequence at the specific region and covered the CpG site at locus cg12620499. In this study, we found that the methylation levels were significantly higher at positions 6 to 9 of CpG sites in the liver tissue when compared with other tissues. Therefore, we increased the specificity of assay by designing a TaqMan MGB probe to complementary with the methylated CpG site at position 6. We could not design the probe to complementary with other CpG sites of this target specific region because of the limitation of TaqMan MGB probe design. For *SHP1P2* gene, the only methylated primer set and probe were chosen according to the previous study (25). In addition, *Beta actin* gene was used to be an internal control for normalizing the amount of DNA sample. The primer set and probe of this gene were derived from the previous study (25) and could determine both methylated and

unmethylated sequences. We combined among three primer sets and probes of these genes to quantify methylated DNA levels of *DSU* and *SHP1P2* genes, which is termed "tissue-specific methylated multiplex real-time PCR technique". In the first step, we optimized condition and validated this technique by detecting methylation profiles in mixed bisulfite treated DNA samples of Huh7, HepG2 and Daudi cell lines. For *DSU* gene, the positive control was bisulfite treated DNA sample of Huh7 and the negative controls were bisulfite treated DNA samples of HepG2 and Daudi. Hepatocytes are the epithelial cells of the liver; therefore, bisulfite treated DNA samples of Huh7 and HepG2 were the positive controls for *SHP1P2* gene. On the other hand, bisulfite treated DNA sample of Daudi was the negative control for this gene because of a hematopoietic cell. We prepared the same DNA concentration of three cell lines before performing the PCR. **Figures 5A 5B and 5C** manifested the ΔRN values of the Beta actin, DSU and SHP1P2 probe signals, respectively. There was found the ΔRN values of Beta actin probe were similar in all cell lines as shown in **Figure 5A**. This result indicated that the amount of three DNA samples were equal. For *DSU* gene, only Huh7 had the ΔRN values of probe signals (**Figure 5B**). This evidence supported the pyrosequencing results and confirmed that this technique was specific for identifying the mature liver cell. It implied that this gene could be an epigenetic marker for hepatocyte. **Figure 5C** displayed that the ΔRN values of SHP1P2 probe in Huh7 and HepG2 were higher than in Daudi. This data confirmed that *SHP1P2* gene could be an epithelial-specific DNA methylation marker. The

validation results showed that this technique and these epigenetic markers would be potential method and epigenetic markers for plasma DNA detection in clinical applications of liver disease. Next step, tissue-specific methylated multiplex real-time PCR technique was utilized to evaluate methylation levels in circulating DNA of patients with liver disease and healthy controls. Final step, we studied the relationship between clinical and pathological characteristics of the liver disease (e.g., stage or grade) and methylation level of each methylation maker.

Baseline characteristics of patients with liver disease and healthy controls

Baseline characteristics of 50 healthy controls, 102 patients with CHB and 121 patients with HBV related-HCC along with clinical implications are summarized in **Table 3** Patients with HBV-HCC were significantly older than healthy controls and CHB. However, there was no difference in mean age and gender distribution among groups of HCC. At initial diagnosis, the HBV-HCC group had higher serum total bilirubin (TB), aspartate aminotransferase (AST), alanine aminotransaminase (ALT) and alkaline phosphatase (ALP) levels but lower serum albumin than the other two groups. There was no significantly difference among the HCC groups in terms of Child-Pugh and BCLC stage.

***DSU* and *SHP1P2* methylation levels in the plasma of patients with liver disease and healthy controls**

To determine the methylation levels of *DSU* and *SHP1P2* in blood sample from patients with CHB, HBV-HCC and healthy controls. Methylated DNA was performed by using real-time PCR with Methylation-Specific primer *Taq*Man probed.

Figure6A indicated that the mean ΔRN value of *DSU* of the healthy controls, patients with CHB and HBV-HCC showed 0.13±0.23, 0.22±0.37 and 0.22±0.37, respectively. The mean ΔRN of *SHP1P2* were 0.06±0.06 for the healthy controls, 0.05±0.05 for CHB and 0.18±0.15 for the HBV-HCC group **(Figure6B)**. These data suggested that methylation levels of *DSU* and *SHP1P2* were significantly higher in patients with HBV-HCC than healthy controls (P=0.045 and P<0.001, respectively), while only *SHP1P2* methylation level was significantly increased in patients with HBV-HCC compared to patients with CHB (P<0.001).

DSU and SHP1P2 methylation levels in subgroup of patients with HCC

To explore whether there was a significant methylation level change of *DSU* and *SHP1P2* in different BCLC stages of HCC. We compared the methylation level in methylated DNA between early stage (0-A) and later stage (B-C). **Figure7A** demonstrated that the methylation level of *SHP1P2* was significantly increased in patients with B-C stage compared with in 0-A stage (0.22±0.17 vs. 0.11±0.08, P<0.001). However, there was no significantly difference between 0-A and B-C stages in *DSU* methylation level (0.27±0.41 vs. 0.23±0.38, P=0.619) **(Figure7B)**.

DSU and SHP1P2 as diagnostic markers for HCC

To differentiate patients with HCC from without HCC, the methylation levels of *DSU* and *SHP1P2* were compared with receiver operating characteristic (ROC) curves. **Figure 8** showed that the area under the curve (AUC) is 0.600 for *DSU* (95%CI: 0.53 to 0.67, P=0.004) and 0.794 for *SHP1P2* (95%CI: 0.74 to 0.85,

P<0.001). The sensitivity and specificity of *DSU* were 72.3% and 45.5% respectively; whereas, the sensitivity and specificity of *SHP1P2* were 61.2% and 84.3% respectively.

DSU and SHP1P2 methylation levels associated with overall survival

To confirm whether these methylation levels can be the potential prognostic in the overall survival of patients with HBV-HCC. The patients were divided into two groups based on the cut-off value of *DSU* and *SHP1P2* methylation levels. Accordingly, there were patients with *DSU* and *SHP1P2* as low (<0.1) and high (≥0.1) of methylation levels groups. The median overall survival (OS) of patients with low and high *DSU* levels were 29 and 27 months (*P*=0.891 by log rank test) (**Figure 9**). Interestingly, patients with low *SHP1P2* methylation level had the median OS at 43 months, which was significantly better than that of patients whose high *SHP1P2* level (median OS, 25 months; *P*=0.018).

In addition, serum *DSU* and *SHP1P2* methylation levels were entered into the univariate and multivariate analysis together with other variable factors. These factors included age, gender, AST, ALT, AFP, BCLC stage, Child-Pugh score, and tumor size. In univariate analysis, factors associated with overall survival consist of AST, ALT, AFP, *SHP1P2* methylation level, BCLC stage, Child-Pugh score and tumor size. In multivariate analysis, only BCLC and Child-Pugh score were independent prognostic factors of overall survival (Table 4).

A

Original sequence

GGGGCTGAGGGACTGGCTGAGAACCGTGTGCTGCTGCTGCGGGTGCGAGTGCTTGGAGGAGCGCGCCCTGCC

TGAGAAGGAGCCCCTCGTCAGGTGGGTGCGCCGCCCGGGCCGGGATCTGGGCTTACCTGGGCCGTAGC

Methylated sequence

GGGGTTGAGGGATTGGTTGAGAATCGTGTGTTGTTGTTGCGGGTGCGAGTGTTTGGAGGAGCGCGTTTTGTTT

GAGAAGGAGTTTTTCGTTAGGTGGGTGCGTCGTTCGGGTCGGGATTTGGGTTTATTTGGGTCGTAGT

Unmethylated sequence

GGGGTTGAGGGATTGGTTGAGAATTGTGTGTTGTTGTTGTGGGTGTGAGTGTTTGGAGGAGTGTGTTTTGTTTG

AGAAGGAGTTTTTTGTTAGGTGGGTGTGTTGTTTGGGTTGGGATTTGGGTTTATTTGGGTTGTAGT

B

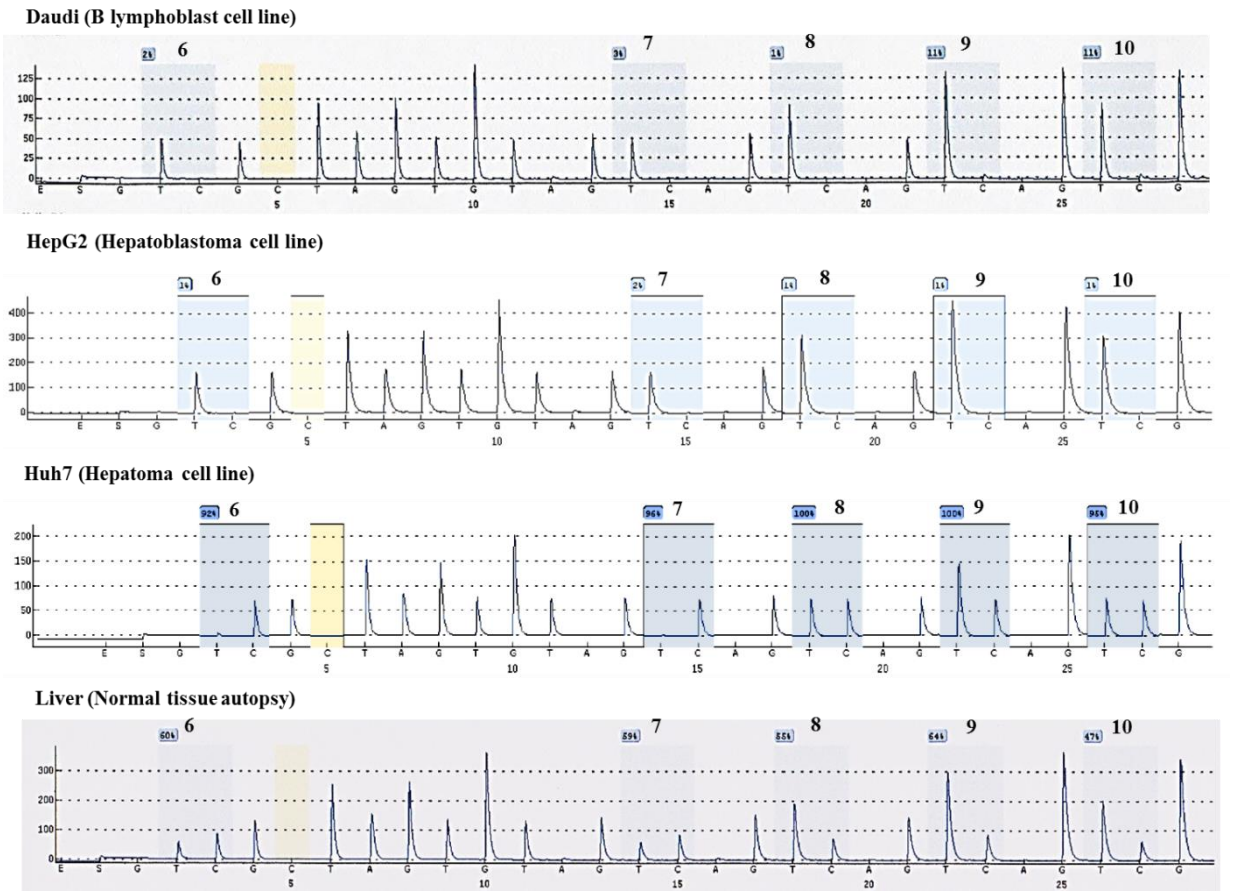

Figure 1. (A) illustrates the partial of original nucleotide sequence of *DSU* gene from Genbank database as compared to the methylated and unmethylated sequences after bisulfite treatment. The underlined sequences are the sequence source,

which is used to design the probe ID Cg12620499 of this gene (ref) and the number indicates each CpG site. The partial pyrograms demonstrate bisulfite pyrosequencing results of DSU2 pyrosequencing primer. In each CpG site, the C-peak or T-peak represents the methylated and unmethylated sequence respectively (B).

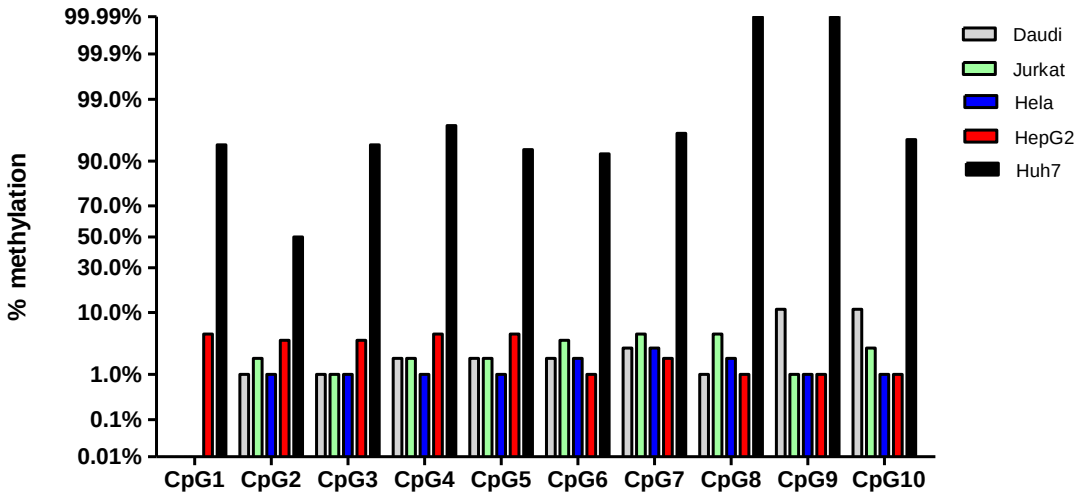

Figure 2. Quantification of percent methylation levels in each CpG site of the *DSU* gene in different cell lines.

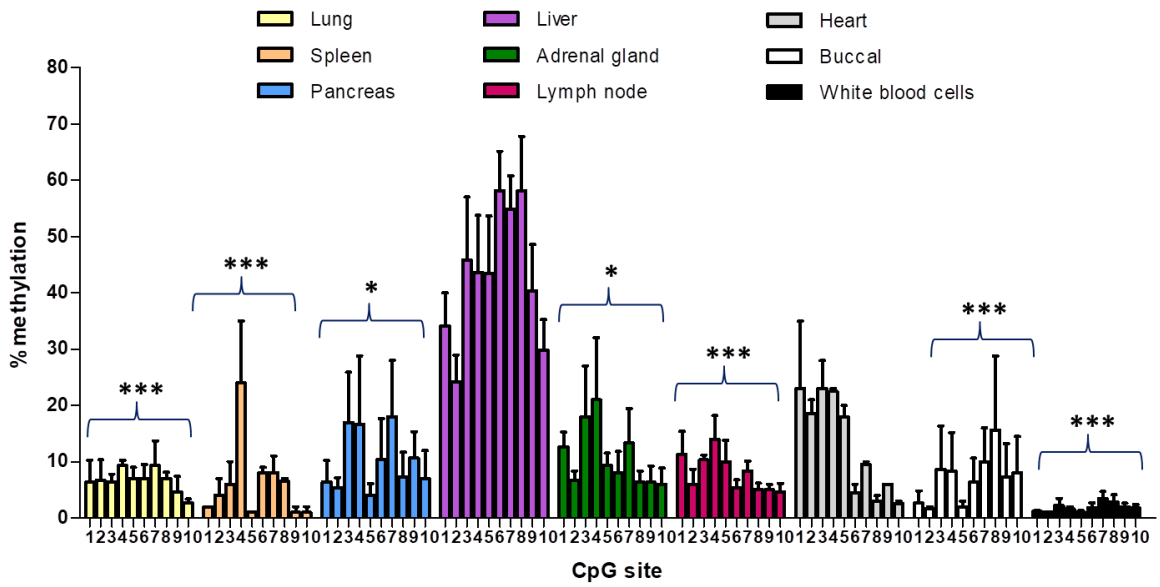

Figure 3. Quantification of percent methylation in each CpG site of the *DSU* gene in different normal tissue types and comparison of average percent methylation levels between liver and each normal tissue type. One asterisk indicates a p-value lower than 0.05, and three asterisks indicate p-value lower than 0.001.

Table 2 Comparison of percent methylation levels in each CpG site between normal liver and other normal tissues

CpG sties / Organs	Lung	Spleen	Pancreas	Adrenal gland	Lymph node	Heart	Buccal	White blood cells
CpG1	P<0.01	P<0.01	P<0.01	NS	NS	NS	P<0.01	P<0.001
CpG2	NS	NS	NS	NS	NS	NS	NS	P < 0.05
CpG3	P<0.001	P<0.001	P<0.01	P<0.01	P<0.001	NS	P<0.001	P<0.001
CpG4	P<0.001	NS	P < 0.05	NS	P<0.01	NS	P<0.001	P<0.001
CpG5	P<0.001	P<0.001	P<0.001	P<0.001	P<0.001	NS	P<0.001	P<0.001
CpG6	P<0.001	P<0.001	P<0.001	P<0.001	P<0.001	P<0.001	P<0.001	P<0.001
CpG7	P<0.001	P<0.001	P<0.001	P<0.001	P<0.001	P<0.001	P<0.001	P<0.001
CpG8	P<0.001	P<0.001	P<0.001	P<0.001	P<0.001	P<0.001	P<0.001	P<0.001
CpG9	P<0.001	P<0.001	P<0.01	P<0.001	P<0.001	P<0.01	P<0.001	P<0.001
CpG10	P < 0.05	P < 0.05	NS	P < 0.05	P < 0.05	P < 0.05	NS	P<0.01

P-values represent the statistical significant difference.

NS is no significance (a p-value higher than 0.05).

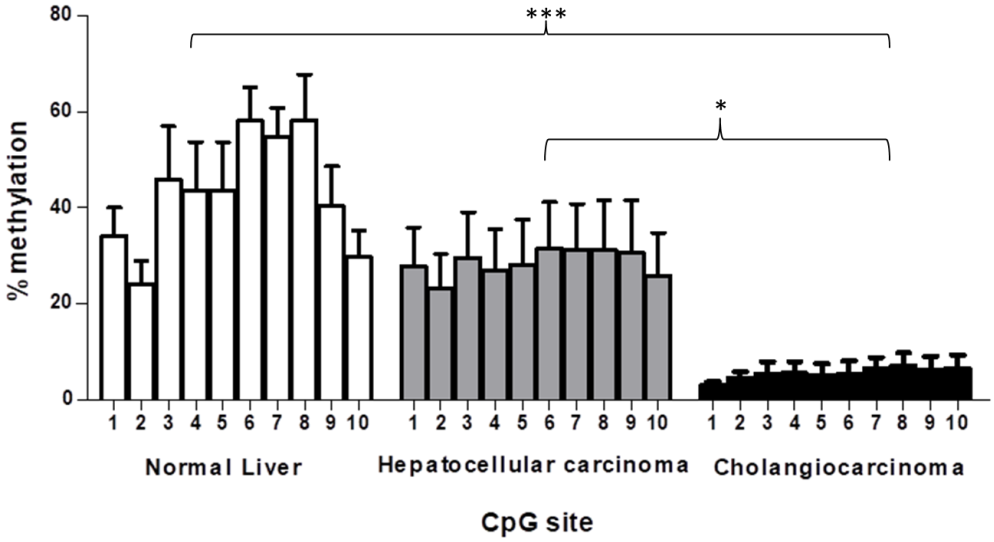

Figure 4. Quantification of percent methylation levels in each CpG site of the *DSU* gene and Comparison of average percent methylation levels among normal liver (tissue autopsy), hepatocellular carcinoma (Tissue biopsy) and Cholangiocarcinoma (Tissue biopsy). One asterisk indicates a p-value lower than 0.05, and three asterisks indicate p-value lower than 0.001.

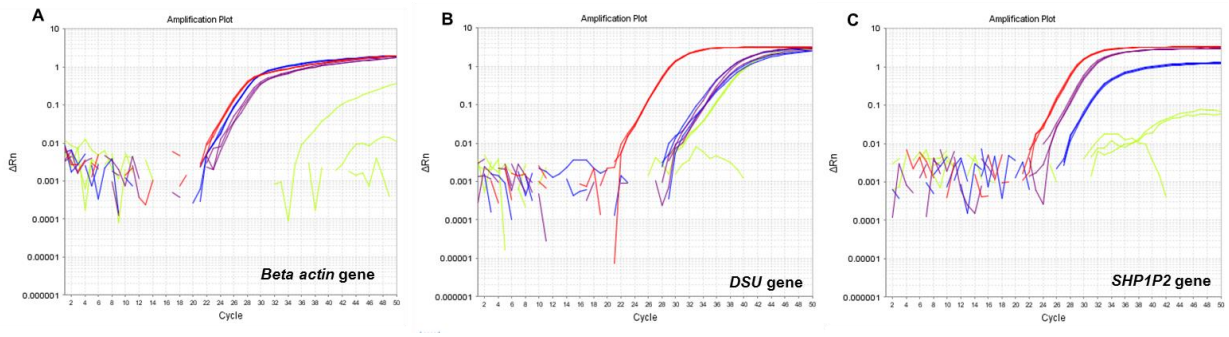

Figure 5. Validation of tissue-specific methylated multiplex real-time PCR technique. (A), (B) and (C) show the ΔRN values of Beta actin, *DSU* and *SHP1P2* probe signals, respectively. Red, Blue and purple represent bisulfite treated DNA samples of Huh7, Daudi and HepG2, respectively. Green is non-template control (water).

Table 3 Baseline characteristics of patients

Characteristics	Healthy Controls (n=50)	CHB (n=57)	CHB-cirrhosis (n=47)	HBV-HCC (n=121)	*P*
Age (years)	46.4±4.6	48.6±11.8	55.9±9.8	60.3±10.9	<0.001*
Sex (%)					
Male	18 (73.3)	223(82.6)	100(76.3)	93 (76.9)	0.194
Female	-26.7	47(17.4)	31(23.7)	28 (23.1)	
TB (mg/dL)		0.8±0.6	0.7±0.4	1.1±0.6	0.001*
Albumin (mg/dL)		4.2±0.5	4.3±0.4	3.5±0.6	<0.001*
AST (IU/L)		28.9±20.6	34.5±20.9	90.5±91.5	0.057
ALT (IU/L)		33.3±42.2	40.2±32.7	53.2±44.3	0.027*
ALP (mg/L)		68.3±23.4	91.2±48.5	162.7±129.5	0.806
AFP (ng/mL)				9,518.8±36,780.2	
Child-Pugh classification					
A				73 (72.9)	-
B-C				30 (27.1)	
BCLC stage					
0-A				40 (33.1)	-
B				41 (33.8)	
C-D				40 (33.1)	
Tumor size (cm)				7.5±5.8	-

Data express as mean ± standard deviation or n (%), Differences between groups were tested by Chi-square test or One-Way ANOVA as appropriate, **P*<0.05, TB: Total bilirubin, AST: aspartate aminotransaminase, ALT: alanine aminotransaminase, ALP: Alkaline phosphatase, AFP: alpha-fetoprotein

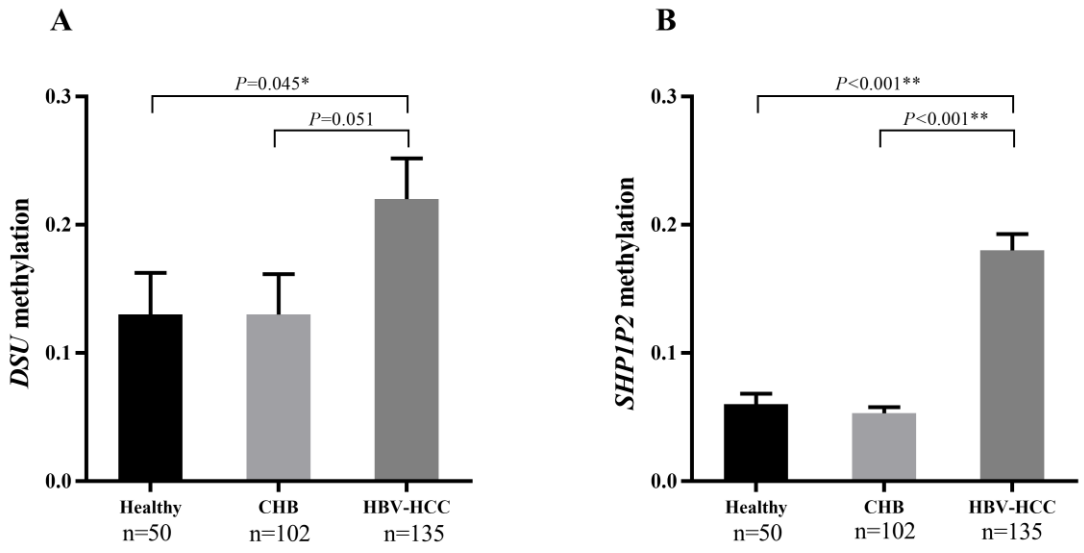

Figure 6. Comparison plasma methylation levels among studied group (A) *DSU* methylation level (B) *SHP1P2* methylation level

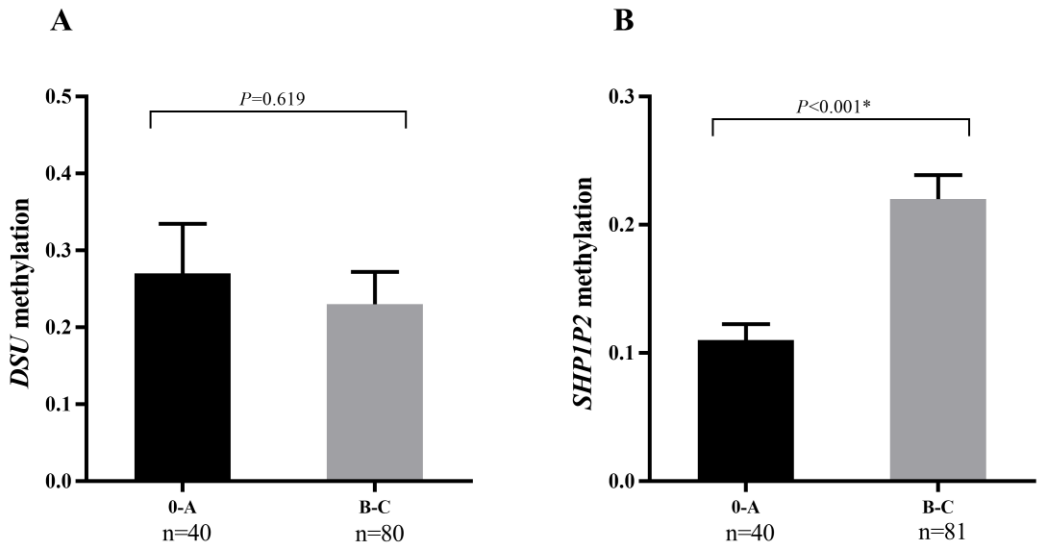

Figure 7. Plasma methylation levels regarding to BCLC stage in patients with HCC (A) *DSU* methylation level (B) *SHP1P2* methylation level

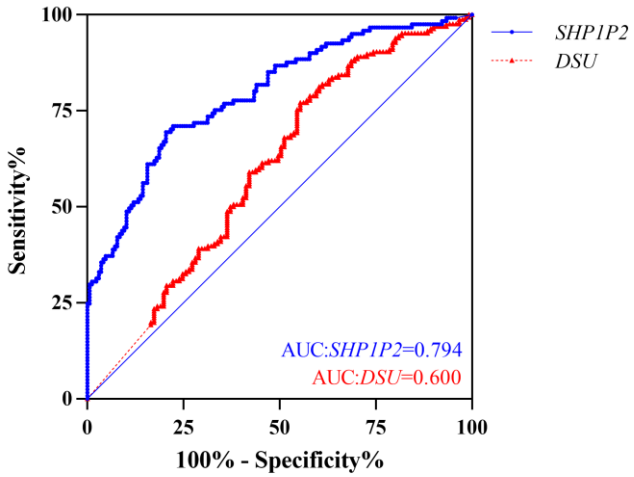

Figure 8 Receiver operating characteristic curves of *DSU* and *SHP1P2* methylation levels

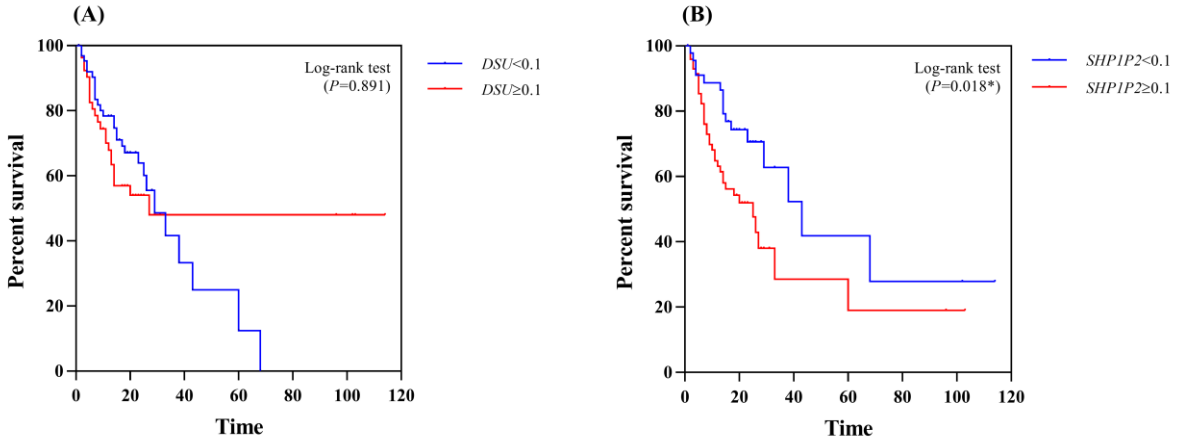

Figure 9. Overall survivals of patients with HCC regarding to methylation levels (A) *DSU* methylation level (B) *SHP1P2* methylation level

Table 4. Factors associated with overall survival in patients with HCC

Factors	Category	Overall survival			
		Univariate analysis		Multivariate analysis	
		OR (95%CI)	*P*	OR (95%CI)	*P*
Baseline					
Age (yr)	< 60 vs. ≥ 60	2.32 (1.31-4.10)	0.004*	1.09 (0.42-2.84)	0.856
Gender	Female vs. Male	0.71 (0.39-1.30)	0.267		
AST (IU/L)	< 60 vs. ≥ 60	3.93 (2.03-7.63)	<0.001*	1.57 (0.41-6.02)	0.510
ALT (IU/L)	< 60 vs. ≥ 60	2.46 (1.38-4.36)	0.002*	1.75 (0.63-4.83)	0.281
AFP (ng/ml)	< 100 vs. ≥ 100	4.13 (1.72-9.94)	0.002*	1.92 (0.68-5.46)	0.220
DSU	<0.1 vs. ≥ 0.1	1.04 (0.60-1.81)	0.892		
SHP1P2	<0.1 vs. ≥ 0.1	2.01 (1.11-3.66)	0.022*	1.12 (0.42-3.01)	0.816
BCLC stages	0-A vs. B-C	6.99 (3.05-16.00)	<0.001*	8.20 (1.37-49.22)	0.021*
Child-Pugh score	A vs. B-C	2.20 (1.22-3.98)	0.009*	4.13 (1.49-11.53)	0.007*
Tumor size (cm)	<5.0 vs. ≥ 5.0	3.93 (2.07-7.47)	<0.001*	0.73 (0.19-2.74)	0.635

Data express as odds ratio (OR), 95% confidence intervals (CI) and were tested by Cox regression analysis;
*, *P*-value<0.05; AST: aspartate aminotransaminase, ALT: alanine aminotransaminase,
ALP: Alkaline phosphatase, AFP: alpha-fetoprotein

Discussion

Circulating nucleic acids (CNA) is currently acceptable to non-invasive and nonspecific biomarker for clinical applications in several diseases. As mentioned above, CNA of healthy people derives from apoptosis of lymphocytes and other nucleated cells. Unlike normal CNA, patients with diseases contain additional cellular sources of circulating DNA resulting from physiological and pathological processes (9). The measurement of circulating DNA level alone is insufficiently sensitive for screening, early detection, diagnosis, staging and prognosis of cancer diseases and is not specific for identification of tumor types. The low levels and small fragments of nucleic acids in bloodstream are also the limitations of CNA detection. For increasing sensitivity and specificity of CNA analysis, next-generation sequencing, real-time PCR, digital droplet PCR and et al are applied to examine genomic mutations, gene expressions and methylation alterations (31). Furthermore, a number of studies have reported that a unique methylation pattern of each cell type can be used to identify the original cell type of DNA segments in blood circulation and body fluid of patients (36). In the present study, tissue-specific methylation markers were applied to identify the original cell type of circulating DNA in patients with liver diseases. Additionally, tissue-specific methylated multiplex real-time PCR technique was developed and validated to escalate sensitivity and specificity of plasma DNA detection. To investigate DSU gene as a hepatocyte-specific methylation marker, DNA methylation pattern of ten CpG sites around position cg12620499 were

examined by pyrosequencing. The hypermethylation status of *DSU* gene was found in Huh7, normal liver tissue and HCC. HepG2 and Huh7 are the cell line derived from hepatoblastoma and HCC, respectively. However, the hypermethylation of all CpG sites were found in Huh7 but not found in HepG2. In general, epigenetic mechanisms regulate several cellular processes including embryogenesis, development, differentiation, X-chromosome inactivation (37), genomic imprinting (38), genomic stability and maintenance of cellular identity (39, 40). Thus, dynamic DNA methylation can occurs during embryonic development. The expression profiles of HepG2 are also approximate to fetal and embryonic hepatoblastomas (35). Several studies present that Huh 7 are differentiated hepatocyte and HepG2 are embryonic hepatocytes. Thus, the hypermethylation of *DSU* gene may be found only in mature hepatocyte. From bioinformatics analysis, our colleagues found high methylation level of CpG at locus 12620499 of *DSU* gene in only liver tissue. This CpG site is the position 5 of ten CpG sites in this study. From our observation, the positions six to nine of all ten CpG sites in liver cells had significant higher methylation levels than other tissue types but not CpG site at the position 5 because of no significant difference between liver and heart tissues. Therefore, the methylated CpG sites at positions six to nine of *DSU* gene may be an appropriate target-specific sequence for identifying hepatocyte from other cell types. Since real-time PCR assay using TaqMan MGB probe was established to detect methylated sequence of *DSU* gene for identifying hepatocyte, our findings demonstrated that this technique was both

sensitive and specific to discriminate the liver cell from other cells. This marker may be a differentiated hepatocyte-specific DNA methylation marker but may not be able to determine fetal and embryonic hepatocytes because the hypermethylation level and fluorescence signal were not found in HepG2. However, further study is required to confirm the methylated sequence of *DSU* gene is specific for differentiated hepatocyte. For *SHP1P2* gene, the fluorescence signals were measured only in Huh7 and HepG2. Our findings confirm that the methylated sequence of *SHP1P2* gene is an epithelial-specific DNA methylation marker, which is in accordance with other investigations (22-25).

After optimization and verification of tissue-specific methylated multiplex real-time PCR technique, we used this method to detect plasma DNA of healthy subjects and patients with liver disease. From our observations, *DSU* gene is improper to be a potential biomarker for clinical applications in HCC. Global hypomethylation and tumor heterogeneity may affect the efficiency of this marker. Epigenetic alterations in cancer can occur in both hypermethylation and global hypomethylation. Both of them lead to tumorigenesis by different pathways. Global hypomethylation is the loss of genomic DNA methylation. A number of studies have reported that global hypomethylation levels in patents with HCC are significant higher than healthy populations (41, 42). Moreover, global hypomethylation levels correlates with poor prognosis and chromosomal instability in patents with HCC (43). In this study, figure 4 illustrated that the liver cancer tissues had lower percentage of methylation levels in

each CpG site of the *DSU* gene than normal liver tissues, although the mean values of percent methylation between the normal and cancer liver were not significantly different. Therefore, demethylation of DNA may appear in *DSU* gene. It may be a cause of reduced methylation level of this gene in HCC patients. Besides global hypomethylation, tumor heterogeneity is different populations of cancer cells within the same primary cancer as well as their metastases from the same patient. Individual cancer cells have different genetic and epigenetic profiles. Consequently, the development of tumor heterogeneity contributes to drug resistance and tumor recurrence after treatment because of clonal evolution. Several studies report that tumor heterogeneity is found in HCC (31, 44, 45). It is possible that the plasma DNA of HCC patients may mix with hypermethylation and hypomethylation of *DSU* gene from different populations of cancer cells. However, further studies are warranted on these issues. Although the methylated CpG site of *DSU* gene is not a potential biomarker for clinical diagnosis of HCC, this marker is a hepatocyte-specific DNA methylation marker. It is able to be a forensic marker for identification of organ tissue type from crime scene because the methylated sequence of *DSU* gene is a specific organ marker but not a specific tumor marker.

On the other hand, *SHP1P2* gene is an efficient biomarker for clinical applications in HCC. This marker is an epithelial-specific DNA methylation marker. A number of studies have utilized this marker to be a biomarker of carcinoma because this cancer type originates in the epithelial tissue that lines the inner or outer surfaces

of the body, such as lung, colon, breast and liver cancers. Therefore, the methylated DNA levels of *SHP1P2* gene have been applied to detect cancer cells in not only body fluids such as plasma and cerebrospinal fluid but also lymph node biopsy. Vinayanuwattikun *et al.* has reported that the advanced non-small cell lung cancer patients had significant high levels of *SHP1P2* methylation in plasma DNA when compared with healthy controls. Moreover, the correlation between increased *SHP1P2* methylation levels and poor prognosis has been found in patients with advanced non-small cell lung cancer (24). In cerebrospinal fluid, *SHP1P2* methylation values have been detected in epithelial-derived malignancy patients with leptomeningeal metastasis but undetected in non-epithelial-derived malignancy patients with leptomeningeal metastasis (46). *SHP1P2* methylation levels have also been found in lymph node tissues for determining metastatic tumors in patients with lung or colorectal cancers (23, 25). Thus, this marker is possible to be a biomarker for identifying the original tissue type but not identifying the specific organ. It is appropriate for use in clinical applications of carcinomas.

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