RESEARCH ARTICLE

Epithelial-Specific SHP1-P2 Methylation - a Novel Universal Tumor Marker for Detection of Colorectal Cancer Lymph Node Metastasis

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Abstract

Background: Methylation of promoter 2 of the SHP1 gene is epithelial cell specific, with reported potential as a lymph node metastatic marker. Objective: To demonstrate *SHP1-P2* methylation-specific quantitative PCR effectiveness in detecting colorectal cancer (CRC) DNA in lymph nodes. Materials and Methods: *SHP1-P2* methylation levels were measured in lymph nodes of CRC patients and compared with pathological findings and patient prognosis. Results: Lymph nodes of CRC metastatic patients without microscopically detectable cancer cells had higher *SHP1-P2* methylation levels than lymph nodes of controls (p<0.001). In addition, a higher *SHP1-P2* methylation level was associated with a shorter duration until adverse disease events, metastasis, recurrence and death (r2 = 0.236 and p value = 0.048). Studying two cohorts of 74 CRC patients without microscopic lymph node metastases showed that only the cohort containing samples with high *SHP1-P2* methylation levels had a significant hazard ratio of 3.8 (95%CI = 1.02 to 14.2). Conclusions: *SHP1-P2* methylation PCR can detect CRC DNA in lymph nodes even if cancer cells are not visible under a microscope, confirming applicability as a potential universal lymph node metastatic marker.

Keywords: *SHP1-P2* - methylation specific epithelial tissue - lymph node metastasis - colorectal cancer

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Introduction

Methylated *SHP1-P2* DNA may be a universal epithelial tumor marker for metastasis in lymph nodes. Promoter 2 of the Src homology region 2 domain-containing phosphatase-1 (SHP1) gene is specifically methylated in epithelial tissue, but not in connective tissue nor in specialized connective tissue (Ruchusatsawat et al., 2006). Therefore, if specialized connective tissue organs, such as blood and lymph nodes, contain methylated *SHP1-P2* DNA, the DNA should be derived from metastasized epithelial cancer cells. Recently, our group proved this hypothesis by reporting the presence of methylated *SHP1-P2* DNA in the plasma and lymph nodes of non-small cell lung cancer patients, while methylated *SHP1-P2* DNA was absent in cancer-free individuals (Vinayanuwattikun et al., 2011; Vinayanuwattikun et al., 2014). Here, we improved methylation specific quantitative PCR and evaluated *SHP1-P2* methylation levels in the lymph nodes of colorectal cancer (CRC) patients. We hypothesized that *SHP1-P2* methylation levels would be higher in metastatic lymph nodes.

Materials and Methods

Study populations and sample collection

This study was approved by the ethical committee IRB NO 476/56 from the Faculty of Medicine, Chulalongkorn University. The study included 805 lymph nodes from 107 CRC patients and 13 tonsillar lymph nodes from 13 healthy individuals. All of the patients were diagnosed at the Chulalongkorn Memorial Hospital between 2003 and 2007. We divided lymph nodes into 4 groups according to the review of two pathologists (SK and NK). The first group was the tonsil node (Normal node: NL). The second and third groups were derived from individual patients who had lymph node metastasis: the second group was lymph nodes without visualized metastatic cancer cells (LP-N), and the third group was lymph nodes with metastatic cancer cells (at least 70% of the lymph node tissue area) (LP-M). The last group was the lymph nodes from colon cancer patients who had no metastasis (LNeg). The LNeg samples were collected from 2 cohorts: the 1st from 2003 to 2007 and the 2nd from 2006 to 2007. For samples selection of each cohort, we selected the lymph

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nodes from CRC patients from 2003 to 2007 by randomly. The 1st was used for preliminary test and the 2nd was used for validating test. All lymph node samples were formalin fixed and paraffin embedded. Each section was cut into a 5-micron section, with 20 sections prepared per sample. Then, sections underwent standard H&E staining for histopathological evaluation.

Control methylated and unmethylated *SHP1-P2* DNA samples were obtained from HEp-2 and Daudi cell lines, respectively, which were purchased from the ATCC and cultured with Dulbecco's modified Eagle's medium and RPMI 1640, respectively. The cell culture media contained 10% fetal bovine serum, and the cells were incubated at 5% CO_2, 37°C and 80% humidity.

DNA extraction and bisulfite treatment

Sections were deparaffinized with a xylene-based protocol, and DNA was extracted using a standard phenol/chloroform technique. Then, 500 micrograms of DNA was treated with bisulfite using EZ DNA methylation-Gold™ kit (Zymo Research Irvine, CA 92614, U.S.A.) according to the manufacturer's instructions.

DNA Sequencing

To characterize methylated and unmethylated *SHP1-P2* amplification products, we performed 2 PCRs using the primers GTT-TTA-TAG-GGT-TGT-GGT-GAG-AAA-TT and ACA-CAT-ATA-TAC-CTT-ACA-CAC-TCC-AAA for each PCR, with bisulfite treated DNA from HEp-2 and Daudi for methylated and unmethylated sets, respectively. Both PCR products were cloned into the pGEM-T Easy vector (Promega, UK) and sequenced.

Combined Methylation-Specific primer TaqMan real-time (COMST) PCR Quantification of *SHP1-P2* methylation levels was performed by a combination of methylation-specific primers and the absolute quantitative TaqMan probe real-time PCR technique. The methylated and unmethylated sets were used to quantitate the amount of methylated and unmethylated DNA. Promoter 2 of the SHP-1 bisulfite oligonucleotide sequences was derived from GenBank accession number U47924.1. For the methylation set, the bisulfite sequences of the forward primer, reverse primer and the probe were

AGGATTTATTCGATGATAGTTGTTATCGTT, CTCCACCAACTACTTTTACGCAAC, FAM-CTAACCCACGCTAATAA-MGB, respectively. The forward primer, reverse primer and probe of the unmethylated set were GTAGGATTTATTTGATGATAGTTGTTATTG, TCCTCCACCAACTACTTTTACACAA and VIC-CCCTAACCCACACTA-MGB, respectively. The two PCR sets were normalized to a bisulfite Beta-actin set, and the bisulfite oligonucleotide sequence was derived from GenBank accession number U01317. The forward primer, reverse primer and probe were GTGTATTTGATTTTTGAGGAGA, CCTTAATACCAACCTACCCAA and Cy5-AAGGTGAAYGTGGATGAAGTTGGTGGTGAGG-BHQ, respectively.

All PCRs were performed as duplex PCR using 2X TaqMan GTXpress real-time PCR master mix (ABI, USA) with an ABI 7500 fast real-time PCR system. The standard curve of each set was generated by serial dilution of bisulfite DNA from HEp-2 and Daudi for methylated and unmethylated sets, respectively. The *SHP1-P2* methylation level was calculated using the following equation: Percentage of *SHP1-P2* methylation=(nM x 100) / (nM + nU)*. *nM (normalized methylated DNA) and nU (normalized unmethylated DNA) is the level of methylated DNA or unmethylated DNA divided by level of beta actin in the same reaction, respectively. The copy number of methylated DNA (M), unmethylated DNA (U) and Beta-actin were from Delta RN (ΔRN was a value of fluorescent signal (RN) from target DNA minus RN from a passive reference dye for a given reaction)of each target was compared with a standard curve generate during varying concentrations of each target.

Statistical analysis

All statistical analyses were carried out using SPSS software version 22 (SPSS Inc., USA). Student's T-test and Mann-Whitney test were used for assessing differences among parametric and non-parametric distributed variables, respectively. For analysis of differences between groups of TNM staging and grading, the Kruskal-Wallis test was used. The Kaplan-Meier method of survival analysis was used to define the time elapsed between case and control groups. The Log-rank test was used to differentiate between case and control groups. For the hazard ratio of relapse-free survival, we used Cox proportion hazards regression analysis. The results of all analyses are reported as odd ratios with a 95% confidence interval. The Pearson correlation coefficient was used to analyze the correlation between methylation level and age. All statistical tests were applied using a significance threshold of $p < 0.05$.

Results

Sequencing of SHP1-P2 methylation

First, we confirmed our previous report (Ruchusatsawat et al., 2006) on the methylation of *SHP1-P2* of epithelial-derived HEp-2 cells and hematological-derived Daudi cells by COMST PCR. Four CpGs were methylated in the PCR product of HEp-2 (Figure1A), and 4 CpGs were unmethylated in the PCR product of Daudi (Figure 1B). Then, we swapped the anti-sense sequencing nucleotide for the sense sequencing nucleotide and then checked with the sequence from the NCBI database (Figure 1C). There were no sequence variations detected from either cell type when compared with the NCBI database (accession number U47924.1).

Validation of COMST Real-time PCR

We invented COMST PCR to be a real-time PCR technique that combined methylation-specific PCR primers (MSP) and a TaqMan probe. This combination mixed the high specificity of MSP PCR and with the high sensitivity of TaqMan probes real-time PCR, giving COMST high reliability, resolution and accuracy. Figures 2A and 2B showed the ΔRN values for the methylated and unmethylated sets, respectively. For the methylated set, there were ΔRN values of methylation probe and beta-actin

probe, and the ΔRN values are shown in a series of 10-fold serial dilutions ranging from 20 ng to2 pg (left to right). For *SHP1-P2* methylation detection, this technique used specific primers with 3 CpGs and 1 CpG for probe to detect methylated and unmethylated *SHP1-P2*, respectively. The precision of *SHP1-P2*methylated and unmethylated set testing was high, and the coefficient of variance (C.V.) of the threshold cycle (Ct) for both sets was measured by

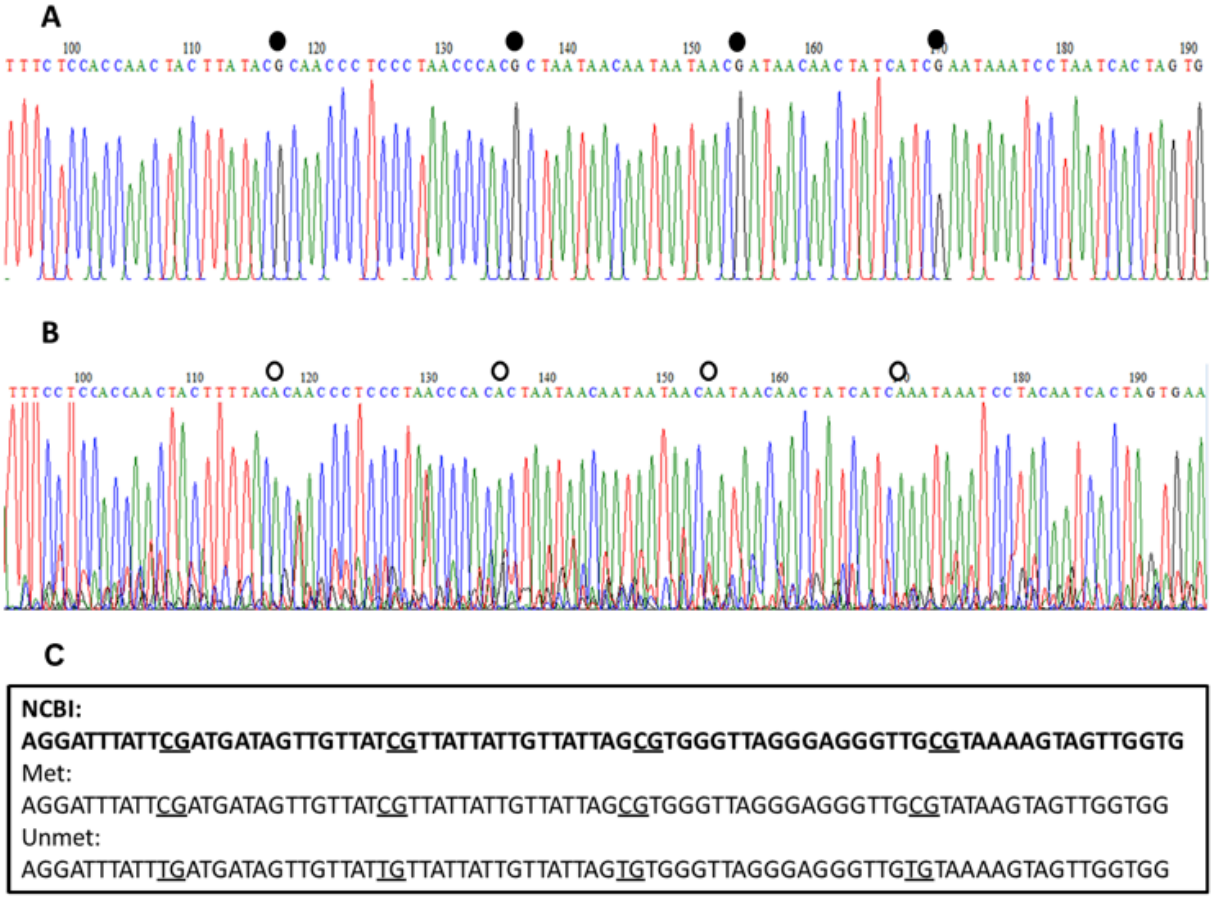

Figure 1. Sequencing Results. (A) and (B) showed the nucleotide sequence of SHP1-P2 methylated and unmethylated, respectively. The sequences are shown in the both DNA strand. The black circles represent the methylated CpGs and the white circles show the unmethylated CpGs. (C) FASTA format nucleotide sequence of sequencing for methylated and unmethylated SHP1-P2 compared to the nucleotide sequence from the NCBI database; the underlined nucleotides represent the studied CpGs

Table 1. Ct Values among Bisulfite DNA of HEp-2 and Daudi Cell Lines

Among of DNA		20ng	2ng	200pg	20pg	2pg	0.2pg	0.02pg
HEp-2	Ct value	25.8 ± 0.1	29.1 ± 0.1	32.8 ± 0.3	35.3 ± 0.4	37.5 ± 1.5	37.7 ± 1.6	37.7 ± 1.8
	C.V.	0.35	0.47	0.87	1.23	3.92	4.32	1.78
Daudi	Ct value	27.6 ± 0.2	30.7 ± 0.1	34.4 ± 0.2	37.4 ± 0.8	39.4 ± 0.6	40.5 ± 0.9	40.2 ± 0.2
	C.V.	0.63	0.48	0.63	2.01	1.53	2.06	0.56

Table 2. Significant Differences between Paira among DNA in HEp-2 and Daudi Cell Lines

Among of DNA	20 ng	2 ng	200 pg	20 pg	2 pg	0.2 pg	0.02 pg
HEp-2	0.02 pg	***	***	***	***	NS	NS
	0.2 pg	***	***	***	***	NS	
	2 pg	***	***	***	***		
	20 pg	***	***	***			
	200 pg	***	***				
	2 ng	***					
	20 ng						
Daudi	0.02 pg	***	***	***	***	NS	NS
	0.2 pg	***	***	***	***	NS	
	2 pg	***	***	***	***		
	20 pg	***	***	***			
	200 pg	***	***				
	2 ng	***					
	20 ng						

* = significant, ** = more significant, *** = the most significant and NS = not significant

performing the PCR on MET and UNMET sets using 10-fold dilutions of HEp-2 and Daudi bisulfite DNA ranging from 20 ng to 0.02 pg and repeated 10 times (Table 1). The C.V. values of varied among the DNA for both sets and ranged from 0.4-5. However, neither set could distinguish the difference in Ct value between 2 and 0.02 pg, so the least among of DNA that could be measured precisely was 2 pg for both sets (Table 2). The weight of total human genomic DNA for 1 cell is approximately 6.67 pg, and the C.V. values of 2 pg of methylated and unmethylated DNA were 0.51 and 1.77, respectively.

Accordingly, this technique was able to detect 1 target cell. The accuracy of the technique was tested by examining the methylation level of mixed plasmids of various methylation levels of *SHP1-P2* and mixing DNA of HEp-2 and Daudi cell lines in various proportions (Table 3). The both correlation were close to linear, and the Pearson's correlation coefficient r2 was 0.9994 and 0.9729, respectively. The technique was accurate in a range between a 1 : 9 and 9 : 1 ratio of HEp-2 : Daudi cells (Table 4), but the values beyond the detection limit were not accurate because there was some background of methylated and unmethylated*SHP1-P2* in the Daudi and HEp-2 cell lines, respectively. This technique was highly precise and accurate to examine the methylation level of *SHP1-P2*. Thus, we used this technique to examine the methylation levels of *SHP1-P2* in lymph nodes from colon cancer patients for the improved detection of micrometastasis.

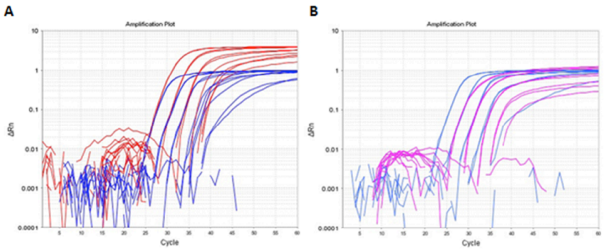

Figure 2. Validation Results. (A) and (B) showed the validation test by Δ of the probe signals of methylation, unmethylation and cloned recombinant plasmid methylated and unmethylated SHP1-P2 amplicons, respectively

Table 3. Accuracy Tests of CoMST PCR. Showed correlation between measured SHP1-P2 methylation level by using CoMST PCR and varied proportions of plasmids (MET : UNMET) and DNA cell lines (HEp-2 and Daudi).

	Plasmid	Cell line
	met : unmet	HEp2 : Daudi
Pearson r	0.9997	0.9863
95% CI	0.9987 to 0.9999	0.9465 to 0.9966
P value	< 0.0001	< 0.0001
R squared	0.9994	0.9729

Micrometastasis detection in lymph nodes from CRC patients

Lymph nodes were collected from 107 CRC patients and separated into positive and negative lymph node groups by diagnosis (Table 5). Two hundred sixty-nine nodes were collected from 33 CRC patients who were diagnosed positive for metastasis in the lymph nodes and separated into 2 groups. The first group contained 141 lymph nodes reviewed for cancer cells under a microscope (LP-M). The median number of nodes per patient was 7 (1-15). The second group (LP-N) contained 128 lymph nodes without identifiable cancer cells under a microscope. A median node per patient was 8 (2-22). The 33 metastatic lymph node CRC patients included 23 males (69.7%) and 10 females (30.3%), and the average age was 61 (27-89) years old. All of the CRC patients in this group were in stage III. For tumor grading, 10 (30.3%) patients had grade I tumors and 21 (63.6%) and 2 (6.1%) patients had tumors of grade II and III, respectively. For other groups, we defined lymph nodes from 74 CRC patients without metastasis into 2 cohorts (1st cohort LNeg, N=37 and 2nd cohort LNeg, N=37). The 1st cohort was divided

Table 4. Differences in SHP1-P2 Methylation Levels with Varied Proportions of DNA cell lines (HEp-2 and Daudi)

	HEp-2 : Daudi						
Proportion	10:00	9:1	7.5:2.5	5:5	2.5:7.5	1:9	0:10
Methylation level	76.8 ± 2.2	69.0 ± 3.4	59.1 ± 2.8	46.9 ± 7	27.3 ± 0.5	2.0 ± 0.3	1.4 ± 0.2
P value		0.0125	0.0004	< 0.0001	0.0084	< 0.0001	0.0205

Table 5. Demographic Data of CRC Patients

Patient Characteristic	Categories	Positive Lymph node		Negative Lymph node		P value
		With CA	Without CA	1st Cohort	2nd Cohort	
Number of patients		33	37	37		
Number of lymph nodes	All nodes	141	128	276	260	N/A
	Nodes per patient	1 to 15	2 to 22	2 to 8	2 to 8	
	Median	7	8	5	5	
Sex	Male	23 (69.7%)		14 (37.9%)	22 (59.5%)	0.852
	Female	10 (30.3%)		23 (62.1%)	15 (40.5%)	
Age (years)		27 - 89		40 - 94	25 - 83	0.2776*
	Mean	61		66	64	
TNM stage	I	0		5 (13.5%)	7 (19%)	
	II	0		32 (86.5%)	30 (81%)	0.6941
	III	33 (100%)		0	0	
Tumor grading	I	10 (30.3%)		23 (62.2%)	24 (64.9%)	
	II	21 (63.6%)		13 (35.1%)	12 (32.4%)	0.452
	III	2 (6.1%)		1 (2.7%)	1 (2.7%)	

P values from Mann-Whitney tests (* p value of Pearson's Correlation).

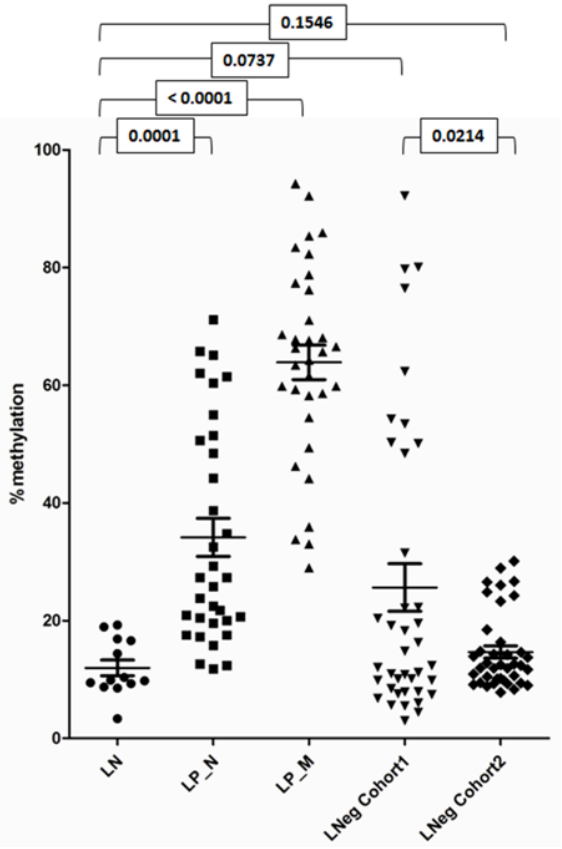

Figure 3. SHP1-P2 Methylation Level of CRC Patients. The SHP1-P2 methylation level of normal node (NL), metastasis lymph nodes without colon cancer cells (LP-N), lymph node metastasis with colon cancer cells (LP-M), negative lymph nodes from the 1st cohort (LNegCohort1) and 2nd cohort (LNegCohort2).The p value of different methylation levels between LN and LP_N was 0.0001, LN and LP_M was less than 0.0001, LN and LNegCohort1 was 0.0737, LN and LNegCohort2 was 0.1546 and LNegCohort1 and LNegCohort2 was 0.0214

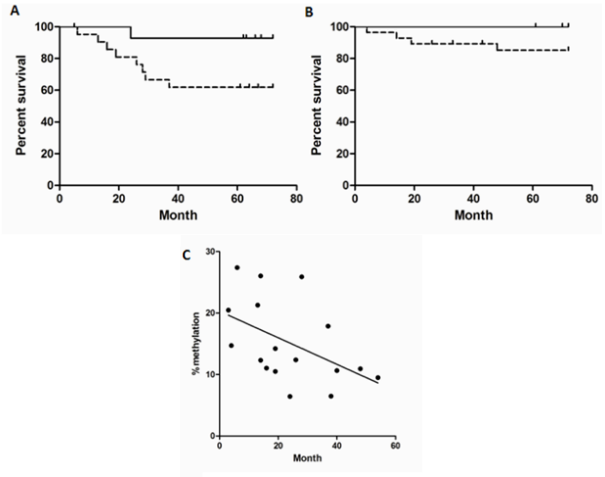

Figure 4. Survival Analysis. (A) and (B) show the Kaplan-Meier model of survival analysis, duration of metastasis, recurrence and death of LN by the dashed line representing the +SHP1-P2 group and the normal line representing the -SHP1-P2 group of the 1st cohort and the 2nd cohort, respectively. (A)The odds ratio of the Log-Rank test was 3.942 (p = 0.0471); the median of +SHP1-P2 and –SHP1-P2 were undefined. The rate of evidence's risk analysis was carried out using the hazard ratio; the hazard ratio was 3.8 (95%CI = 1.02 to 14.2). (C) The relationship between SHP1-P2 methylation level and times (months) of evidence, recurrence and/or death in the LNeg group (1st + 2nd cohort) (r^2 = 0.236 and p value = 0.048)

into 276 nodes. The median node per patient was 5 (2-8). The average age of the 14 (37.9%) males was 61 (32-89) years old and of the 23 (62.1%) females was 62 (41-78) years old. TNM staging classification of CRC patients in this group were stage I and II for 5 (13.5%) and 32 (86.5%) patients, respectively. Regarding tumor grading classification, the number of patients having tumors of grade I was 23 (62.2%), of grade II was 13 (35.1%) and of grade III was 1 (2.7%). For the 2nd cohort, the lymph nodes in were divided into 260 nodes. The median node per patient was 5 (2 -8) and the mean age was 64 (25-83) years old. For TNM staging classification of CRC patients in this group, were in stage I and for 7 (19%) and 30 (81%), respectively. Regarding tumor grading classification, the number of patients having tumors of grade I was 24 (64.9%), of grade II was 12 (32.4%) and of grade III was 1 (2.7%).

Thirty-three lymph nodes from CRC patients who had metastasis and thirteen tonsil nodes from healthy individuals were reviewed and confirmed by two expert pathologists. The lymph nodes from metastatic CRC patients were divided into 2 groups: the first group was lymph nodes without histologically identifiable cancer cells (LP-N), and the other group was lymph nodes with cancer cells (LP-M). The tonsil nodes were the normal group (NL). In addition, only lymphatic tissue was carefully dissected from the tonsil nodes to avoid contamination of epithelial tissue from the outer layer of the tonsil node. For LP-M, we conducted *SHP1-P2* methylation level analysis of lymph nodes with colon cancer cell metastasis occupying up to approximately 70% of the section. The *SHP1-P2* methylation level is shown in Figure 3, comparing that of LP-M with NL; the methylation level of LP-M was higher than NL and highly significant (p<0.0001), with the mean methylation level of LP-M and NL being 63.89 ± 2.928 and 11.96 ± 2.928%, respectively. Interestingly, *SHP1-P2* methylation levels in the LP-N group were also significantly higher than those of the NL group (p=0.0001) (Figure 3). The mean methylation level of LP-N was 34.14 ± 3.231, suggesting the LP-N group contained undetectable epithelial DNA without detectable cancer cells under the microscope.

Next, we evaluated *SHP1-P2* methylation levels in the LNeg group (Figure 3). The *SHP1-P2* methylation level of the LN group was lower than that of the LNeg group of the 1st and 2nd cohorts (the mean methylation level of cohort 1st LNeg was 25.00 ± 4.17% and 2nd cohorts LNeg was 14.85 ± 1.08%). There was a significant different between LNeg of the 1st and 2nd cohorts (p=0.0214). Therefore, the 1st cohorts contained more micrometastasis in the nodes than the 2nd cohort.

For survival analysis, we studied the 2 cohorts (Figure 4).There was no significant difference in *SHP1-P2* methylation level between different genders, ages, stages and tumor grades (Table 1). We divided the *SHP1-P2* status of negative lymph nodes into 2 groups using a cut-off *SHP1-P2* methylation level of 10%. The positive

SHP1-P2 (+*SHP1-P2*) had *SHP1-P2* methylation levels of 10% or higher, and the negative *SHP1-P2* (-*SHP1-P2*) had *SHP1-P2* methylation levels lower than 10%. The cut-off point of *SHP1-P2* methylation level at 10% was analyzed using the Log-Rank test and selecting the best P value of the odds ratio. The Kaplan-Meier method model of the 1st cohort demonstrated significant differences in the odds ratio of the Log-Rank test was 3.942 (p=0.0471), and the medians of the +*SHP1-P2* group and the –*SHP1-P2* group were undefined (Figure 4A). The rate of evidence's risk analysis was carried outusing the hazard ratio, and the hazard ratio was 3.8 (95%CI=1.02 to 14.2). For the 2nd cohort, the survival rate of the +SHP-P2 group was also lower than that of the –*SHP1-P2* group, but the difference was not statistically significant (Figure 4B). These results were expected because the 1stcohort contained more patients with cancer DNA in lymph nodes than the 2nd cohort. In addition, we combined both cohorts to evaluate the relationship between *SHP1-P2* methylation level and time of death and recurrence in the LNeg group (Figure 4C). We found that the high *SHP1-P2* methylation level had a higher evidence of risk than the low *SHP1-P2* methylation level (r^2=0.236 and p value=0.048). Therefore, the more cancer DNA identified in lymph node, the poorer the prognosis would be.

Discussion

The COMST PCR technique was highly sensitive in detecting and measuring DNA methylation level. We separated methylated and unmethylated DNA detection into two tubes due to three reasons. First, unmethylated DNA measurement in the same probe reduced detection sensitivity. Second, unmethylated DNA detection experiment can be used as validation of methylated DNA detection. Finally, in some study set up direct measurement of unmethylated DNA may be needed (Vinayanuwattikun et al., 2013). Nevertheless, we found the same information when applying only methylated DNA detection in one tube experiment. The COMST PCR technique can detect up to single-cell levels of DNA (2-20 pg, (1 human cell contains approximately 6.77 pg of DNA)) with high precision, distinguishing DNA methylation levels.

COMST PCR to measure *SHP1-P2* in lymph nodes from CRC patients is an efficient method for identifying micrometastasis. First, the *SHP1-P2* methylation levels of histologically negative lymph nodes of CRC patients with evidence of metastasis were higher than those of normal controls. Second, the *SHP1-P2* methylation levels of node negative CRC patients were higher in cases of recurrence and/or poorer prognosis.

Lymph node micrometastasis are a problem resulting in the underdiagnosis and conservative TNM classification of cancer patients. There have been a number of molecular techniques attempted to detect lymph node micrometastasis, such as evaluation of cancerous DNA, RNA or protein from occult tumor cells (Kitkumthorn et al., 2012; Patel et al., 2013). However, the effectiveness of such techniques has yet to be satisfactory. Here, we used *SHP1-P2* methylation PCR detected CRC cancer DNA in the lymph nodes. By improved the PCR sensitivity the measurement can detect cancer DNA even if cancer cells were not visible under a microscope.

To be considered as universal lymph node metastatic marker, epithelial-specific methylation possesses advantages over promoter hypermethylation of tumor suppressor genes. Unlike epithelial-specific methylation, there is epigenetic heterogeneity of promoter hypermethylation of tumor suppressor genes in which each tumor methylates tumor suppressor genes differently (Patel et al., 2003; Anglim et al., 2008; Brock et al., 2008; Payne et al., 2009; Kim et al., 2010). Each tumor methylates different tumor suppressor genes. For example, P16 and CDH13 are methylated in NSCLC at 34 and 27% but methylated in CRC at 28 and 65% (Brock et al., 2008; Kim et al., 2010; Vinayanuwattikun et al., 2014). Therefore, a combination of this type of tumor marker is necessary to improve the sensitivity of the test. Many studies focused on developing the techniques to detect methylation levels of selected tumor suppressor gene promoters for each cancer cell (Patel et al., 2003). However, there was a report that found that the hypomethylation level of lymph node tissue was a main cause of corruption of the hypermethylation level measurement of metastatic tumor cells (Huynh and Hoon). Moreover, for practical purposes, promoter methylation of tumor suppressor genes of primary tumors is always required before evaluating the methylation level of tumor suppressor genes in metastatic tumor cells (Toyooka et al., 2001; Safar et al., 2007; Chujan et al., 2014; Tiwawech et al., 2014). In contrast, for epithelial-specific methylation detection, *SHP1-P2* is ubiquitously methylated in epithelial cells and unmethylated in connective tissue (Ruchusatsawat et al., 2006). Therefore, sensitivity and specificity using even a single epithelial marker for detecting epithelial cancer cells in lymph nodes is very high. Additionally, there is no need to test methylation status in the primary sites.

In conclusions, COMST is a new methylation specific quantitative PCR approach with high sensitivity and specificity. Here, we confirmed that *SHP1-P2* DNA methylation is universal lymph node metastatic markers by evaluating lymph nodes from CRC patients. This PCR method measures higher levels of *SHP1-P2* methylation in lymph nodes from CRC patients with evidence of metastasis or recurrence. Therefore, COMST PCR for *SHP1-P2* methylation is a highly sensitive and specific technique in detecting micrometastasis of epithelial-derived cancer in specialized connective tissues.

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