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Brief communication (original)

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Evaluation of *SHP1-P2* methylation as a biomarker of lymph node metastasis in patients with squamous cell carcinoma of the head and neck

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Abstract

Background: Hypermethylation of *Src* homology region 2 domain-containing protein-tyrosine phosphatase 1 promoter 2 (*SHP1-P2*) has been proven as an epithelial-specific marker. This marker has been used for the detection of lymph node metastasis in patients with lung cancer or colon cancer.

Objectives: To investigate *SHP1-P2* methylation in patients with squamous cell carcinoma of the head and neck (HNSCC) and determine its potential for micrometastasis detection in the lymph nodes of patients with HNSCC.

Methods: *SHP1-P2* methylation levels were analyzed by combined methylation-specific primer TaqMan real-time PCR in 5 sample groups: normal tonsils (n = 10), microdissected squamous cell carcinoma epithelia (n = 9), nonmetastatic head and neck cancer lymph nodes (LN N0, n = 15), metastatic HNSCC histologically negative for tumor cells (LN–, n = 18), and matched cases histologically positive for tumor cells (LN+, n = 18).

Results: *SHP1-P2* methylation of 10.27 ± 4.05% was found in normal tonsils as a lymphoid tissue baseline, whereas it was 61.31 ± 17.00% in microdissected cancer cell controls. In the 3 lymph node groups, the *SHP1-P2* methylation levels were 9.99 ± 6.61% for LN N0, 14.49 ± 10.03% for LN– Nx, and 41.01 ± 24.51% for LN+ Nx. The methylation levels for LN– Nx and LN+ Nx were significantly different ($P = 0.0002$). Receiver operating characteristic curve analysis of *SHP1-P2* methylation demonstrated an area under the curve of 0.637 in distinguishing LN N0 from LN– Nx.

Conclusions: *SHP1-P2* methylation was high in HNSCC, and low in lymphoid tissues. This methylation difference is concordant with lymph node metastasis.

Keywords: DNA methylation; lymph nodes; neoplasm micrometastasis; promoter regions, genetic; squamous cell carcinoma of head and neck; *Src* homology region 2 domain-containing protein-tyrosine phosphatase 1

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Squamous cell carcinoma of the head and neck (HNSCC) is a common form of cancer with high mortality, and the number of cases is increasing annually [1]. HNSCC can be fatal, frequently from metastasis. In cases of single unilateral lymph node metastasis, the 5-year survival rate is less than 50%, and in cases of bilateral metastases, the survival is less than 25% [2]. In general, histological biopsy is the criterion standard for HNSCC metastasis detection in lymph nodes, but this is uncomfortable for patients and requires an experienced pathologist for assessment. Moreover, limitations, such as inadequate tissue sampling and oversights in detecting minute tumor cells, make identification of metastatic HNSCC difficult. This may lead to underdiagnosis and inaccurate tumor staging, resulting in inappropriate therapeutic management [3–7]. Thus, a biomarker to detect metastatic HNSCC and replace the traditional histological method is highly desirable. The *Src* homology region 2 domain-containing protein-tyrosine phosphatase 1 promoter 2 (*SHP1-P2*) is specifically methylated in epithelial tissue and unmethylated in other tissues, including blood vessels, nerves, fibroblasts, and white blood cells [8–10]. In addition, methylated *SHP1-P2* DNA has been observed in cancer cells originating from epithelial cells [9, 11, 12]. Hence, if methylated *SHP1-P2* DNA is present, it is likely to be derived from metastasized epithelial cancer cells.

Recently, our group reported the presence of methylated *SHP1-P2* DNA in the plasma and lymph nodes of patients with nonsmall-cell lung cancer, whereas methylated *SHP1-P2* DNA was absent in cancer-free individuals [9, 11]. Later, we developed an efficient method for the measurement of *SHP1-P2* methylation, we called combined methylation-specific primer TaqMan real-time (COMST) polymerase chain reaction (PCR) [12]. This method can detect colorectal cancer (CRC) DNA in lymph nodes even if cancer cells are not visible under a microscope, suggesting its potential for detecting markers of epithelial cancer metastasis in lymph nodes.

In the present study, we aimed to determine whether measurement of *SHP1-P2* methylation can be used as an effective biomarker to detect metastatic HNSCC and whether these measurements can provide support for diagnosing nodal metastases from HNSCC as a possible alternative to routine histopathology.

Materials and methods

Sample inclusion

The present study was approved by the Institutional Review Board (IRB) of the Faculty of Medicine, Chulalongkorn University, Thailand (IRB No. 516/56, approval No. 004/2014). All samples were coded-anonymized formalin-fixed, paraffin-embedded tissue retrieved retrospectively from the Department of Pathology, Faculty of Medicine, Chulalongkorn University, affiliated with King Chulalongkorn Memorial Hospital, a tertiary care, university teaching hospital in central Bangkok, Thailand. The samples included 10 normal tonsils, and samples from 51 patients diagnosed with HNSCC including 9 samples of primary HNSCC and 42 lymph nodes from patients with and without HNSCC metastasis as inclusion criteria. With the exception of one 11-year-old boy, all other patients were in their fourth decade or older at the time of clinical sampling. Lymph nodes had been derived from radical neck dissections and were classified into 3 types: (1) lymph nodes from patients with control cases of nonmetastatic HNSCC (LN N0, n = 15), (2) lymph nodes from patients with cases of metastatic HNSCC, but histologically negative for carcinoma cells (LN– Nx, n = 18), and (3) lymph nodes from patients with matched cases of metastatic HNSCC that were histologically positive for carcinoma cells (LN+ Nx, n = 18). The normal tonsils and the LN N0 and LN– Nx types were confirmed histologically to be free of carcinoma cells, whereas lymph nodes of the LN+ Nx type were diagnosed as having metastatic malignant cells in at least 70% of the nodal tissue. All samples were confirmed as meeting the criteria by consensus from 2 pathologists (NK and SK). The demographic data of the patients, clinical stages, and histological grades were reviewed from their medical records and are presented in **Table 1**.

DNA extraction and bisulfite modification

All formalin-fixed, paraffin-embedded (FFPE) tissues were sliced into 3 to 5 sections of 5 μm thickness and left unstained. Another section was stained with hematoxylin and eosin (HE) for histopathological confirmation. In the HNSCC groups, tumor cells were manually microdissected as previously reported [13]. In brief, FFPE tissues were cut serially into 5 levels. The first and the last of the 5 slides were stained with HE. Selected areas on the first slide were circled using a marker pen. Subsequently selected areas on the last slide were also marked using the first slide as a reference and subsequently examined under a microscope. If the last slide was correctly marked, the remaining unstained slides (levels 2–4) would be processed in the same manner using the first and last HE-stained slides as references to select the areas. Later, the marked areas on the unstained slides were dissected using a standard 21-gauge needle. After deparaffinization with xylene, the DNA was isolated using Tris-buffered sodium

Table 1. Demographic data of patients with squamous cell carcinoma of the head and neck (HNSCC)

Sex	Age (y)	Organ	Histological grade	Clinical stage
Microdissected HNSCC				
M	51	Floor of mouth	2	2
F	62	Tongue	1	1
M	65	Floor of mouth	2	3
F	62	Tongue	3	1
M	67	Gingiva	1	2
M	50	Gingiva	1	2
M	61	Tongue	2	4
M	37	Tongue	2	2
M	56	Tongue	3	4
N0 (no metastasis)				
11	M	Tongue	2	4
67	F	Buccal mucosa	1	3
47	F	Tongue	1	2
53	F	Tongue	1	1
51	M	Floor of mouth	2	3
83	F	Lip	2	4
70	M	Soft palate	1	3
48	F	Soft palate	1	4
72	M	Palate	2	2
38	F	Tongue	2	2
46	F	Tongue	1	1
65	M	Buccal mucosa	2	3
81	M	Tongue	2	1
72	F	Buccal mucosa	2	2
63	M	Tongue	2	1
Nx (metastasis)				
52	M	Tongue	1	1
57	M	Soft palate	3	3
54	M	Soft palate	2	4
56	M	Tongue	2	4
79	F	Palate	3	4
74	M	Buccal mucosa	1	4
42	M	Tongue	3	4
66	M	Soft palate	2	4
51	M	Tongue	2	4
66	F	Buccal mucosa	1	2
58	M	Tongue	2	4
67	M	Tongue	2	4
60	M	Buccal mucosa	1	4
69	M	Gingiva	2	4
34	M	Tongue	1	4
64	F	Tongue	2	3
61	M	Soft palate	2	3
84	F	Lower lip	2	4

Histological grade: 1 = well-differentiated, 2 = moderately-differentiated, 3 = poorly-differentiated

dodecyl sulfate with proteinase K and then left at 50°C overnight, followed by phenol–chloroform extraction and ethanol precipitation. The isolated genomic DNA was eluted and then used for bisulfite treatment. Bisulfite modification of the genomic DNA was performed using an EZ DNA Methylation Kit (Zymo Research). The bisulfite-treated DNA was resuspended in 20 μL of water and stored at –20°C until needed for use.

COMST PCR

Quantification of *SHP1-P2* methylation levels was performed using a combination of methylation-specific primers and the absolute quantitative TaqMan probe real-time PCR technique, as previously published [12]. Briefly, the methylated and unmethylated sets were used to quantitate the amount of methylated and unmethylated DNA, respectively. The methylation set contained 5′-AGGATTTATT CGATGATAGTTGTTATCGTT-3′ as the forward primer, 5′-CTCCACCAACTACTTTTACGCAAC-3′ as the reverse primer, and 5′-FAM-CTAACCCACGCTAATAA-MGB-3′ as the probe. The unmethylated set comprised 5′-GTAGGATT TATTTGATGATAGTTGTTATTG-3′ as the forward primer, 5′-TCCTCCACCAACTACTTTTACACAA-3′ as the reverse primer, and 5′-VIC-CCCTAACCCACACTA-MGB-3′ as the probe. The 2 PCR sets were normalized with the β-actin control set, which was composed of the forward primer 5′-GTGTATTTGATTTTTGAGGAGA-3′, the reverse primer 5′-CCTTAATACCAACCTACCCAA-3′, and the probe 5′-Cy5-AAGGTGAAYGTGGATGAAGTTGGTGGTGAGG-BHQ-3′.

All PCRs were performed as duplex PCR using 2× TaqMan GTXpress Real-Time PCR Master Mix (Applied Biosystems) with an ABI 7500 fast real-time PCR system. The *SHP1-P2* methylation level was calculated using the following equation:

$$\text{Percentage of } SHP1\text{-}P2 \text{ methylation} = (\text{nM} \times 100)/(\text{nM} + \text{nU})$$

where nM (normalized methylated DNA) and nU (normalized unmethylated DNA) are the levels of methylated DNA or unmethylated DNA, respectively, divided by the level of β-actin in the same reaction. The copy numbers for methylated DNA, unmethylated DNA, and β-actin were obtained by comparing the change in the fluorescence signal (ΔRN) of the target DNA minus the RN from a passive reference dye for a given reaction of each target with a standard curve generated using varying concentrations of each target. To determine whether *SHP1-P2* methylation is epithelial carcinoma cell specific, tonsil tissue and microdissected HNSCC epithelia were used as negative and positive controls, respectively.

Statistical analyses

All statistical analyses were conducted using IBM SPSS Statistics for Windows (version 22). Descriptive data for the *SHP1-P2* methylation levels are expressed as mean ± standard deviation. Independent sample Student *t* tests were conducted to determine the differences between all groups except for LN– Nx and LN+ Nx, for which a paired Student *t* test was applied. $P < 0.05$ was considered as significant. A receiver operating characteristic curve analysis was conducted to assess the ability of the *SHP1-P2* methylation level to differentiate between lymph nodes with or without metastases.

Results

Comparison of *SHP1-P2* methylation levels in tonsils and microdissected HNSCC epithelia

The level of *SHP1-P2* methylation from the tonsil samples was 10.27 ± 4.05% (n = 10) compared with 61.31 ± 17.00% (n = 9) in primary microdissected HNSCC epithelia (**Figure 1A**).

SHP1-P2 methylation level in the 3 lymph node groups

The *SHP1-P2* methylation in LN N0, LN– Nx, and LN+ Nx was 10.27 ± 4.05%, 14.49 ± 10.03%, and 41.01 ± 24.51%, respectively. A significant difference was observed between LN N0 and LN+ Nx. In addition, the paired *t* test showed a significant difference ($P < 0.0001$) between LN– Nx and LN+ Nx (**Figure 1A** and **B** and **Table 2**). There were no significant differences in *SHP1-P2* methylation levels between different sex, ages, stages, and tumor grades (data not shown).

Evaluation of *SHP1-P2* methylation level to detect HNSCC micrometastases in lymph nodes

Comparing the *SHP1-P2* methylation levels for LN N0 and LN– Nx, the percent *SHP1-P2* methylation yielded a maximal area under the curve at 0.637. This value could be used detect occult carcinomas with a cutoff value of 10.14%, sensitivity of 66.67%, and specificity of 60% (**Figure 1C**).

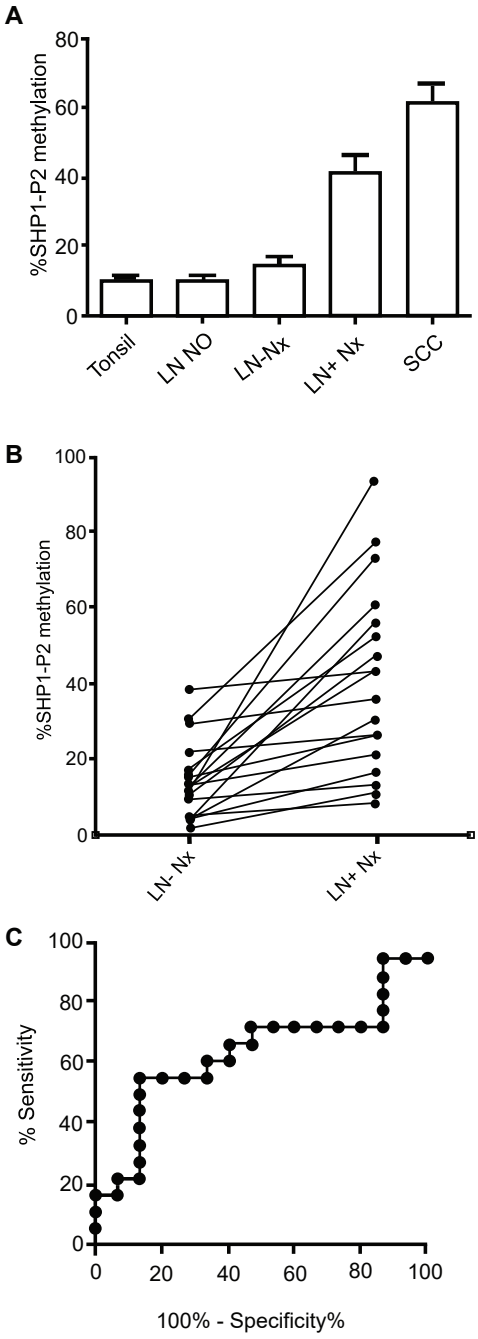

Figure 1. *SHP1-P2* methylation. (**A**) Percentage of *SHP1-P2* methylation comparisons in the 5 sample groups. (**B**) Comparison of LN– Nx and LN+ Nx. A significant difference was observed between LN– Nx and LN+ Nx ($P < 0.0001$). (**C**) ROC curve analysis to distinguish LN N0 from LN– Nx. The curve showed AUC = 0.637 with a sensitivity of 66.67% and a specificity of 60%. AUC, area under the curve; HNSCC, squamous cell carcinoma of the head and neck; LN N0, nonmetastatic HNSCC lymph nodes; LN–Nx = lymph nodes that are histologically negative for tumor cells in patients with metastatic HNSCC; LN+ Nx, lymph nodes that are histologically positive for tumor cells in metastatic HNSCC; ROC, receiver operating characteristic; SCC, squamous cell carcinoma; *SHP1-P2, Src* homology region 2 domain-containing protein-tyrosine phosphatase 1 promoter 2 patients with

Discussion

Metastatic HNSCC has a high mortality and a low cure rate [14]. Various methods have been developed for detecting

Table 2. Percentage of *SHP1-P2* methylation

Group	n	*SHP1-P2* methylation (%, mean ± SD)
Tonsil	10	10.27 ± 4.05
LN N0	15	9.99 ± 6.61
LN– Nx	18	14.49 ± 10.03
LN+ Nx	18	41.01 ± 24.51
SCC	9	61.31 ± 17.00

HNSCC, squamous cell carcinoma of the head and neck; LN N0, nonmetastatic HNSCC lymph nodes, LN– Nx, lymph nodes that are histologically negative for tumor cells in patients with metastatic HNSCC; LN– Nx, lymph nodes that are histologically positive for tumor cells in patients with metastatic HNSCC; SCC, squamous cell carcinoma; *SHP1-P2*, *Src* homology region 2 domain-containing protein-tyrosine phosphatase 1 promoter 2

micrometastatic tumor cells or DNA, including serial section staining, immunohistochemistry, and PCR or reverse transcription-PCR of various genes [15–19]. However, the existing methods are inconclusive and do not provide satisfactory results. At present, immunohistochemistry with a cytokeratin antibody is used widely [20]. However, it is very difficult to detect only a minute HNSCC cell in the lymph node and sometimes it is not possible to see any HNSCC cell by observing just 1 section. Molecular techniques are more sensitive than immunohistochemistry, and can detect HNSCC DNA without tumor cell detection [18, 21].

DNA methylation is a common epigenetic event in cancer and plays an important role in tumor progression [22, 23]. *SHP1-P2* is a tissue-specific gene. *SHP1-P2* methylation is proven to be inversely correlated with expression in epithelial cells [8]. Because there is no report of *SHP1-P2* mutation, *SHP1-P2* methylation is considered responsible for *SHP1-P2* expression. Here we observed *SHP1-P2* methylation; methylated *SHP1-P2* DNA is epithelial cell specific and may be used as a universal epithelial tumor marker for metastasis in lymph nodes. In our previous study of CRC, COMST PCR was used to detect *SHP1-P2* methylation levels in CRC metastasis in lymph nodes without microscopically detectable cancer cells and the methylation levels were found to be higher than those in the nonmetastatic CRC lymph nodes ($P < 0.001$) [12]. The COMST PCR technique is sufficiently sensitive to detect the DNA from a single epithelial cell and has been shown to have a high precision in distinguishing epithelial cells from other cell types based on their DNA methylation level [12].

The results of this study showed that *SHP1-P2* promoter methylation is significantly different in the tonsil group (organ that contains only lymphoid tissue) compared with that in microdissected HNSCC epithelia (totally epithelial cancer cells). Thus, this technique is highly sensitive for detecting HNSCC cells. The *SHP1-P2* methylation level in LN N0 was the same as that in the tonsil group as the tissue types are the same. The case-matched evaluation of patients with LN+ Nx compared to LN– Nx demonstrated significantly higher *SHP1-P2* methylation in LN+ Nx. These results confirmed the concordant effect of HNSCC cells in the lymph nodes. Then, we assessed this marker for its applicability to HNSCC micrometastasis detection using the LN– Nx samples as representatives. Unfortunately, we did not find a significant difference in the levels of *SHP1-P2* methylation between LN N0 and LN– Nx. Therefore, this marker is far from conclusive for HNSCC micrometastasis detection, which might be attributed to the possibility that the LN– Nx could be at a high level in normal lymphocytes with very little or no HNSCC DNA in the sample for examination. Furthermore, *SHP1-P2* methylation levels were not correlated with the clinical stage or histological grade (data not shown), which is consistent with previous findings [12, 21].

A cutoff value of 10.14% was chosen when considering both highest levels of sensitivity and specificity level. At this suitable value, we were able to develop a useful test for distinguishing lymph nodes that are metastatic tumor positive with a sensitivity of 66.7% and specificity of 60%. Therefore, we do not recommend that *SHP1-P2* methylation be used for confirmation; histopathological examination should remain the criterion standard for a definitive diagnosis. Our findings also suggest the potential use of this technology as an ancillary tool for detecting micrometastatic tumors in lymph node metastases, in cases where tumor cells are not detected by routine pathological examination. The sensitivity and specificity of using the single epithelial marker for detecting HNSCC cells in lymph nodes is promising. In future work, we suggest investigating the use of *SHP1-P2* methylation in combination with other biomarkers, such as long interspersed nuclear element-1 and Alu methylation [21]. Given the limited sample size, evaluation of a larger population should also be performed.

Author contributions. NK and AM substantially contributed to the conception and design of the study. SK, JW, and PR substantially acquired the data, and NK analyzed and interpreted it. NK and PR drafted the manuscript. SK, JW, and AM critically revised it. NK, SK, JW, and AM approved the final version submitted for publication and take responsibility for the statements made in the published article; NK as corresponding author takes responsibility for PR in this regard.

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Conflict of interest statement. With the exception of Prakasit Rattanatanyong, for whom the corresponding author takes responsibility, the authors have each completed an International Committee of Medical Journal Editors Uniform Disclosure Form for Potential Conflicts of Interest. None of the authors has any potential conflict of interest to disclose.

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