

## RESEARCH ARTICLE

# LINE-1 and Alu Methylation Patterns in Lymph Node Metastases of Head and Neck Cancers

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## Abstract

**Background:** The potential use of hypomethylation of Long INterspersed Element 1 (LINE-1) and Alu elements (Alu) as a biomarker has been comprehensively assessed in several cancers, including head and neck squamous cell carcinoma (HNSCC). Failure to detect occult metastatic head and neck tumors on radical neck lymph node dissection can affect the therapeutic measures taken. **Objective:** The aim of this study was to investigate the LINE-1 and Alu methylation status and determine whether it can be applied for detection of occult metastatic tumors in HNSCC cases. **Methods:** We used the Combine Bisulfite Restriction Analysis (COBRA) technique to analyse LINE-1 and Alu methylation status. In addition to the methylation level, LINE-1 and Alu loci were classified based on the methylation statuses of two CpG dinucleotides in each allele as follows: hypermethylation (<sup>m</sup>C<sup>m</sup>C), hypomethylation (<sup>u</sup>C<sup>u</sup>C), and 2 forms of partial methylation (<sup>m</sup>C<sup>u</sup>C and <sup>u</sup>C<sup>m</sup>C). Sixty-one lymph nodes were divided into 3 groups: 1) non-metastatic head and neck cancer (NM), 2) histologically negative for tumor cells of cases with metastatic head and neck cancer (LN), and 3) histologically positive for tumor cells (LP). **Results:** Alu methylation change was not significant. However, LINE-1 methylation of both LN and LP was altered, as demonstrated by the lower LINE-1 methylation levels ( $p < 0.001$ ), higher percentage of <sup>m</sup>C<sup>u</sup>C ( $p < 0.01$ ), lower percentage of <sup>u</sup>C<sup>m</sup>C ( $p < 0.001$ ) and higher percentage of <sup>u</sup>C<sup>u</sup>C ( $p < 0.001$ ). Using receiver operating characteristic (ROC) curve analysis, %<sup>u</sup>C<sup>m</sup>C and %<sup>m</sup>C<sup>u</sup>C values revealed a high level of AUC at 0.806 and 0.716, respectively, in distinguishing LN from NM. **Conclusion:** The LINE-1 methylation changes in LN have the same pattern as that in LP. This epigenomic change may be due to the presence of occult metastatic tumor in LN cases.

**Keywords:** Long INterspersed element-1s (LINE-1s) - Alu elements - DNA methylation - lymph nodes - occult tumor

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## Introduction

DNA methylation of the human cancer genome is usually lower (hypomethylation) than in representative normal cells (Chalitchagorn, 2004; Hoffmann, 2005; Esteller, 2011; Kitkumthorn, 2011). The methylated CpG dinucleotides in 5'UTR of the two most abundant interspersed repetitive sequences, Long INterspersed Element-1 (LINE-1 or L1) and Alu elements, are frequently evaluated as representative of the genome-wide methylation levels. Genome-wide hypomethylation, which is characterized by reduced methylation levels of LINE-1 and Alu, is often present in many malignancies (Kitkumthorn, 2011). The epigenomic changes are associated with genomic instability and altered gene expression (Hoffmann, 2005; Kongruttanachok, 2010; Aporn Dewan, 2011). Both LINE-1 and Alu hypomethylation are also associated with advanced tumor stage, higher histological grade, poorer prognosis and

tumor metastasis (Cho, 2007; Choi, 2007; Shuangshoti, 2007; Tangkijvanich, 2007; Pattamadilok, 2008; Iramaneerat, 2011; Kitkumthorn, 2011; Nopavichai, 2012).

Each LINE-1 locus has a distinct methylation level and pattern (Phokaew, 2008; Singer, 2012). To improve the efficiency of detecting cancer DNA and to measure the overall methylation level, we recently classified LINE-1s into 4 groups based on the methylation statuses of 2 CpG dinucleotides in each LINE-1 sequence. These 4 classes were hypermethylation (<sup>m</sup>C<sup>m</sup>C), hypomethylation (<sup>u</sup>C<sup>u</sup>C), and 2 forms of partial methylation (<sup>m</sup>C<sup>u</sup>C and <sup>u</sup>C<sup>m</sup>C) (Pobsook, 2011; Kitkumthorn, 2012). We found that differences in the LINE-1 methylation pattern could be observed even if the overall levels were unchanged. Furthermore, partial methylation contributed to differences in the overall methylation levels between various normal tissue types, oral epithelium and white blood cells (Pobsook, 2011). Finally, for cancer DNA

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detection, %<sup>5</sup>C<sup>4</sup>C is more sensitive and specific than the methylation level (Pobsook, 2011; Kitkumthorn, 2012).

Head and neck squamous cell carcinoma (HNSCC) is one of the most serious health problems, ranking as the sixth most common cancer worldwide (Bennett, 2008). Metastasis from these cancers can be fatal (Manikantan, 2012). The 5-year survival rate is less than 50% for patients with a single unilateral lymph node metastasis and less than 25% for patients with bilateral metastases (Zhang, 2007). Genome-wide hypomethylation has also been found to be involved in the carcinogenesis of these cancers. In a limited cohort of 6 tumor samples, we demonstrated LINE-1 hypomethylation in HNSCC versus histologically normal mucosal tissues (Chalitchagorn, 2004). This result has been confirmed by Smith et al., who determined that 67% of HNSCCs are hypomethylated compared to normal mucosal specimens (Smith, 2007). Furthermore, the latter study also demonstrated a relationship between increasing tumor stage and the degree of hypomethylation—that is, the mean levels of genome-wide methylation are reduced in the Stage IV lesions compared to the Stage I-III diseases (Smith, 2007). Moreover, in a recent study, we found that tumors at metastatic sites presented significant decreases in methylation compared with the primary lesions (Nopavichai, 2012).

Despite the important role of pathologists in cancer-staging evaluations (one of which is the diagnosis of lymph node metastasis), there are some limitations of the routine histological examination for identifying occult metastatic tumor cells. Failure to detect occult tumor cells may be due to inadequate tissue sampling or the inattention of pathologists to minuscule tumor-cell groups. These actions can cause inaccurate tumor staging and lead to improper therapeutic management (Gu, 2002; Coello, 2004; Imoto, 2006; Broglie, 2011; Rahbari, 2012). Many studies have developed methods for detecting occult tumor cells or DNA, including serial section staining, immunohistochemistry, PCR and RT-PCR-based methods (Tsavellas, 2001; Riethdorf, 2008; Wada, 2008; Shimizu, 2012); however, their conclusions have been less satisfactory. In this study, we aimed to determine whether LINE-1 and Alu methylation measurements are effective biomarkers for the detection of occult metastatic HNSCC and whether these measurements can provide a supportive method for diagnosing nodal metastases from HNSCC as an alternative to routine pathological examination.

## Materials and Methods

### Recruited subjects

Sixty-one lymph nodes were included in this study. All specimens were retrieved from the Department of Pathology, Faculty of Medicine, Chulalongkorn Hospital. All of the primary cancer tissue was collected from patients with diagnosed squamous cell carcinoma. Lymph node samples were derived from radical neck dissections and were classified into 3 groups: 1) lymph nodes from cases with non-metastatic head and neck cancer (NM, n=15), 2) lymph nodes from cases with metastatic head and neck cancer but histologically negative for tumor cells (LN,

n=23), and 3) lymph nodes from cases with metastatic head and neck cancer and histologically positive for tumor cells (LP, n=23). The NM and LN groups were confirmed to be histologically free of cancer cells, whereas the LM group was diagnosed as having metastatic malignant cells by two pathologists (NK and SK). The lymph nodes in groups 2 and 3 (LN and LM) belonged to the same group of patients. The patients' demographic data, clinical stages and histological grades were reviewed from each patient's chart and are presented.

### DNA extraction and bisulfite modification

Formalin-fixed, paraffin-embedded lymph node tissues were sliced into 3-5 sections (each of them 5  $\mu$ m thick) and were then left unstained. Another section was stained with hematoxylin and eosin for pathological confirmation. After deparaffinization with xylene, the DNA was isolated using Tris/SDS and proteinase K and 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 previously published methods (Chalitchagorn, 2004). In brief, 200 ng of DNA was dissolved in 50  $\mu$ l of distilled water, followed by denaturation in 5.5  $\mu$ l of 2 M NaOH for 10 min at 37°C. Then, 30  $\mu$ l of 10 mM hydroquinone (Sigma-Aldrich, Singapore) and 520  $\mu$ l of 3 M sodium bisulfite (pH 5.0) were added, mixed and incubated at 50°C for 16 h. The bisulfite-treated DNA was purified using the Wizard DNA clean-up kit (Promega, Madison, WI) according to the manufacturer's protocols. After this step, the DNA was eluted with 50  $\mu$ l of warm water, desulfonated with 5.5  $\mu$ l of 3 M NaOH for 5 min and precipitated (with NH<sub>4</sub>OAc-EtOH) using glycogen as a carrier. Finally, the bisulfite-treated DNA was resuspended in 20  $\mu$ l of water and stored at -20°C until needed for use.

### Combined Bisulfite Restriction Analysis (COBRA) of LINE-1 and Alu

For COBRALINE-1, the bisulfite-treated DNA was subjected to 40 PCR cycles with LINE-1-F (5'-CCGTAAGGGGTTAGGGAGTTTTT-3') and LINE-1-R (5'-RTAAAACCCTCCRAACCAATATAAA-3') primers at an annealing temperature of 50°C. For COBRAAlu, the bisulfite-treated DNA was subjected to 40 cycles of PCR with two primers, Alu-F (5'-GGCGCGGTGGTTTACGTTTGTAA-3') and Alu-R (5'-TTTACCATATTAACCAAAC-3') at an annealing temperature of 53°C. After PCR amplification, the LINE-1 amplicons (160 bp) were digested with TaqI and TasI in NEB buffer 3 (New England Biolabs, Ontario, Canada) while the Alu amplicons (117 bp) were digested with TaqI in TaqI buffer (MBI Fermentas, Burlington, Canada). Both digestion reactions were incubated at 65°C overnight. The LINE-1- and Alu-digested products were then electrophoresed on an 8% non-denaturing polyacrylamide gel and stained with the SYBR green nucleic-acid gel stain (Gelstar, Lonza, USA). The intensities of both COBRA PCR fragments were measured using a phosphoimager

with ImageQuant Software (Molecular Dynamics, GE Healthcare, Slough, UK). Distilled water was used as a negative control. DNA samples from HeLa, Jurkat, and Daudi cell lines were used as positive controls in every experiment to standardize the interassay variation. All experiments were performed in duplicate.

#### LINE-1 methylation analysis

The COBRALINE-1 amplicons generated 4 bands based on the methylation status of the 2 CpG dinucleotides as follows:  ${}^m\text{C}^m\text{C}$ , 160 bp;  ${}^u\text{C}^u\text{C}$ , 98 bp; 1 methylated CpG ( ${}^m\text{C}$ , 80 bp); and 1 unmethylated CpG ( ${}^u\text{C}$ , 62 bp) (Figure 1A). The LINE-1 methylation level of each pattern was calculated according to previous methods (Kitkumthorn, 2012) to determine the precise percentage of CpG dinucleotides.

Briefly, the calculation was performed using the following steps. First, the intensity of each band was separated by the length (bp) of the double-stranded DNA as follows:  $\%160/160=A$ ,  $\%98/94=B$ ,  $\%80/78=C$  and  $\%62/62=D$ . Second, the LINE-1 methylation levels were computed using the following formulas: percentage of overall methylated loci ( $\%{}^m\text{C}$ )= $100\times(C+A)/(C+2A+B+D)$ ,  $\%{}^m\text{C}^m\text{C}=100\times((C-D+B)/2)/(((C-D+B)/2)+D+A)$ ,  $\%{}^u\text{C}^u\text{C}=100\times B/(((C-D+B)/2)+A+D)$ ,  $\%{}^m\text{C}^u\text{C}=100\times(A)/(((C-D+B)/2)+A+D)$  and  $\%{}^u\text{C}^m\text{C}=100\times(D-B)/((C-D+B)/2)+A+D)$ .

#### Alu methylation analysis

The COBRAAlu produced 3 bands according to the methylation status:  ${}^u\text{C}^u\text{C}$ , 117 bp; two partially methylated sequences,  ${}^m\text{C}^u\text{C}$  and  ${}^u\text{C}^m\text{C}$  (74 and 75 bp, respectively); and methylated loci ( ${}^m\text{C}$ , 42 and 43 bp) (Figure 1B). The

Alu methylation level of each pattern was calculated to obtain the exact percentage number.

The calculation was performed as follows: Initially, the intensity of each band was divided by the length (bp) of the double-stranded DNA:  $\%117/117=A$ ,  $\%74$  and  $75/74.5=B$ ,  $\%42$  and  $43/43.5=D$ , and  $D-B=C$  ( $C$ =hypermethylated loci,  ${}^m\text{C}^m\text{C}$ ). Then, the Alu methylation level in each pattern was calculated as follows:  $\%{}^m\text{C}=100\times(2C+2B)/(2A+2B+2C)=100\times(2D)/(2A+2D)$ ,  $\%{}^m\text{C}^m\text{C}=100\times C/(A+B+C)$ ,  $\%{}^u\text{C}^u\text{C}=100\times A/(A+B+C)$  and percentage of partially methylated loci ( $\%{}^u\text{C}^m\text{C}+\%{}^m\text{C}^u\text{C}$ )= $100\times B/(A+B+C)$ .

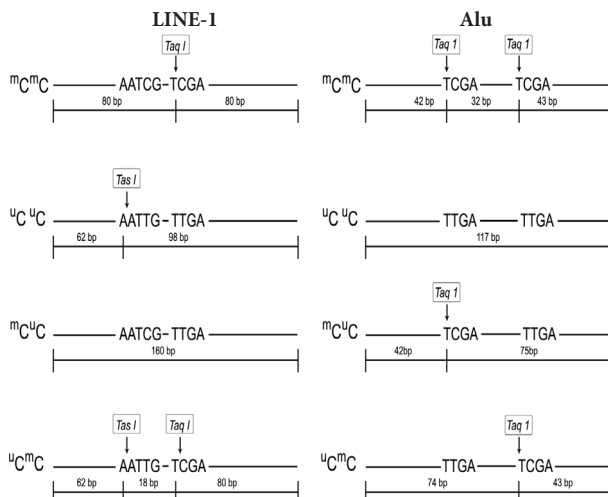
#### Statistical Analysis

All statistical analyses were conducted using SPSS software for Windows version 17.0 (SPSS Inc., Chicago, IL). An independent sample t-test was performed to determine the difference between the NM and LN groups and between the NM and LM groups, whereas the paired t-test was used to evaluate the LN and LM groups. All p-values were two sided. The p-values that were less than 0.05 were considered statistically significant. A receiver operating characteristic (ROC) analysis was performed to verify the ability of the COBRALINE-1 and COBRAAlu methylation status to differentiate between lymph nodes with or without occult metastatic tumor.

## Results

### Overall LINE-1 and Alu methylation

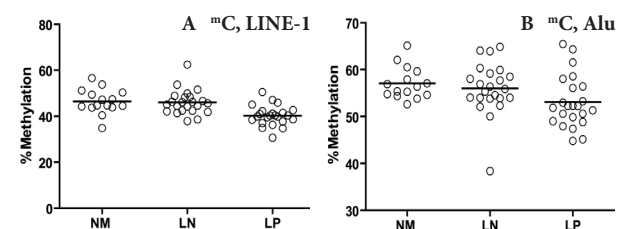
As shown in Table 1, Table 1 and Figure 2, the percentage of overall LINE-1 methylation ( ${}^m\text{C}$ ) among



**Figure 1. LINE-1 and Alu Methylation Patterns.** COBRALINE-1 and COBRAAlu possess four possible methylation patterns: hypermethylated loci ( ${}^m\text{C}^m\text{C}$ ), hypomethylated loci ( ${}^u\text{C}^u\text{C}$ ), and 2 partially methylated loci ( ${}^m\text{C}^u\text{C}$  and  ${}^u\text{C}^m\text{C}$ ). TaqI is a restriction enzyme that is specific to methylated cytosine, whereas TasI is specific to unmethylated cytosine. a) The various methylation patterns of the digested LINE-1 PCR product yielded four differently sized digested products of 160 bp, 98 bp, 80 bp and 62 bp. b) The various methylation patterns of the Alu PCR products yielded four differently sized digested products of 117 bp, 74/75 bp, 42/43 bp and 32 bp.

**Table 1. Each Pattern and p-value**

	$\%{}^m\text{C}$	$\%{}^m\text{C}^m\text{C}$	$\%{}^m\text{C}^u\text{C}$	$\%{}^u\text{C}^m\text{C}$	$\%{}^u\text{C}^u\text{C}$
LINE-1 methylation levels (mean $\pm$ S.D.) and p-value:					
NM (N=15)	46.42 $\pm$ 5.38	22.86 $\pm$ 9.22	20.03 $\pm$ 6.44	27.10 $\pm$ 11.57	30.01 $\pm$ 9.21
LN (N=23)	46.01 $\pm$ 5.13	22.89 $\pm$ 6.70	27.17 $\pm$ 5.70	19.08 $\pm$ 7.78	30.86 $\pm$ 5.65
LP (N=23)	40.21 $\pm$ 4.31	19.88 $\pm$ 5.81	32.09 $\pm$ 6.38	9.21 $\pm$ 8.10	39.47 $\pm$ 6.78
p-value:					
NM vs. LN	0.817	0.991	0.001	0.016	0.728
NM vs. LP	<0.001	0.278	<0.001	<0.001	0.001
LN vs. LP	<0.001	0.136	0.007	<0.001	<0.001
Alu methylation levels (mean $\pm$ S.D.) and p-value:					
NM (N=15)	57.09 $\pm$ 3.42	22.74 $\pm$ 5.67	34.35 $\pm$ 6.53	42.91 $\pm$ 3.42	
LN (N=23)	56.01 $\pm$ 5.49	18.45 $\pm$ 6.43	37.56 $\pm$ 5.62	43.99 $\pm$ 5.49	
LP (N=23)	53.08 $\pm$ 5.56	15.13 $\pm$ 9.83	38.36 $\pm$ 7.42	46.92 $\pm$ 5.56	
p-value					
NM vs. LN	0.498	0.042	0.116	0.498	
NM vs. LP	0.017	0.010	0.097	0.017	
LN vs. LP	0.073	0.112	0.636	0.073	



**Figure 2. Comparison between the Percentages of the Overall LINE-1 and Alu Methylation Levels Among the NM, LN and LP Groups.** Black horizontal bars represent the mean. ' ${}^m\text{C}$ ' represents the overall levels of methylation.

the 3 groups demonstrated a stepwise decrease from NM to LN to LP, respectively. The p-values of the differences between NM and LP and between LN and LP were <0.001. However, when compared between NM and LN, the p-value was insignificant (p=0.817). The same trend was also found in Alu, i.e., the overall methylation level (<sup>m</sup>C) among the 3 groups tended to decline from NM to LN to LP, respectively, though a significant difference was only observed between NM and LP (p=0.017).

*Percentage of LINE-1 methylation in each pattern*

The level of each LINE-1 methylation pattern and its p-value are presented in Table 1 and Figure 3. The value of %<sup>m</sup>C did not reveal significant differences among the 3 groups. However, %<sup>u</sup>C increased stepwise from NM to LN to LP; significant differences were found between NM and LP (p=0.001) and between LN and LP (p<0.001). Interestingly, the levels of partial methylation were significantly different among the 3 groups. The value of %<sup>m</sup>C progressively increased from NM to LN to LP. The predictive value of the differences between each pair was as follows: NM:LN (p=0.001), NM:LP (p<0.001) and LN:LP (p=0.007). In contrast, %<sup>u</sup>C decreased stepwise from NM to LN to LP, with significant differences between NM and LN (p=0.016), NM and LP (p<0.001), and LN and LP (p<0.001).

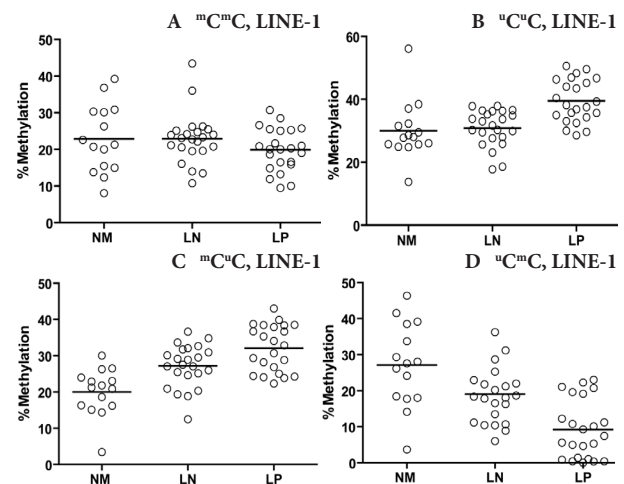
*Alu methylation in each pattern*

The level of each Alu methylation pattern is presented in Table 3 and Figure 4. Similar to LINE-1, the percentage of <sup>m</sup>C decreased stepwise from NM to LN to LP. The difference was statistically significant between NM and LN (p=0.042) and between NM and LP (p=0.010), but the methylation rate between LN and LP was insignificant (p=0.112). In contrast, although the percentage of <sup>u</sup>C seemed to gradually increase from NM to LN to LP, the difference was only significant between NM and LP (p=0.017). Furthermore, no significant differences in the percentage of partially methylated Alu loci were observed.

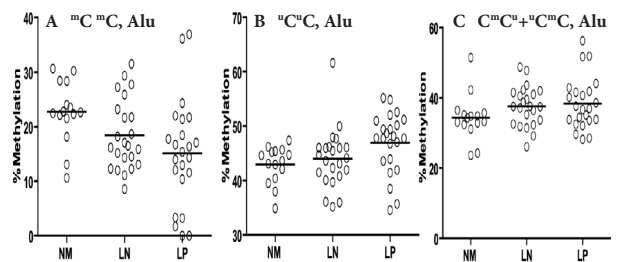
*Application of the levels of partially methylated LINE-1 loci to differentiate between the LN and NM groups*

Next, we evaluated the benefit of this test for the detection of occult tumor. We selected some significant data points to develop a test by setting an optimal cut-off value and using it for the calculation of the sensitivity, specificity and area under the curve (AUC). Among the various patterns of LINE-1 and Alu methylation, %<sup>u</sup>C and %<sup>m</sup>C values of LINE-1 methylation had the highest potential to distinguish the LN group from the NM group (Figures 5A, 5C). The %<sup>m</sup>C value yielded the maximal AUC at 0.716. This pattern could detect the occult tumor with a cut-off value of 22.94%, sensitivity of 78.26% and specificity of 66.67% (Figure 5B). Whereas, %<sup>u</sup>C yielded the highest AUC at 0.806, with a cut-off value, sensitivity and specificity of 23.98%, 78.3% and 80.0%, respectively (Figure 5D). We also determined whether the combination of these two markers could improve the diagnostic power for the detection of occult tumor. In the case of screening tests such as fine needle aspiration (FNA), the test criteria of higher %<sup>m</sup>C or lower

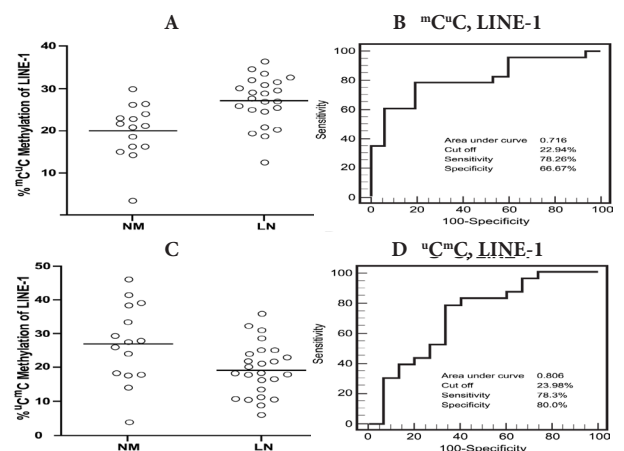
%<sup>u</sup>C exhibited a higher sensitivity (95.30%) but lower specificity (53.30%). In contrast, using the combination of higher %<sup>m</sup>C and lower %<sup>u</sup>C for the detection of occult tumor made the test more specific (93.30%) but less sensitive (61.28%).



**Figure 3. Comparison between the Percentages of LINE-1 Methylation Levels in Each Pattern Among the NM, LN and LP Groups.** Black Horizontal bars Represent the Mean. ‘<sup>m</sup>C’ and ‘<sup>u</sup>C’ Represent the Hyper- and Hypomethylated LINE-1 Loci, Respectively; ‘<sup>m</sup>C’ and ‘<sup>u</sup>C’ Represent the Partially Methylated LINE-1 Loci.



**Figure 4. Comparison between the Percentages of Alu Methylation Levels Among the NM, LN and LP Groups.** Black horizontal bars represent the mean. ‘<sup>m</sup>C’ and ‘<sup>u</sup>C’ represent the hyper- and hypomethylated Alu loci, respectively; ‘<sup>m</sup>C+<sup>u</sup>C’ represents the total partially methylated Alu loci.



**Figure 5. LINE-1 Methylation Patterns can Distinguish the LN Group From the NM Group.** A) %<sup>m</sup>C between the NM and LN groups. B) ROC curve analysis of %<sup>m</sup>C of LINE-1 methylation and cancer detection. C) %<sup>u</sup>C between the NM and LN groups. D) ROC curve analysis of %<sup>u</sup>C of LINE-1 methylation and cancer detection.



## Discussion

DNA methylation is one of the most commonly occurring epigenetic events in the mammalian genome and has demonstrated an important role in tumor progression (Kitkumthorn, 2011). These changes affect different types of repetitive DNA sequences, such as retrotransposons, endogenous retroviruses and satellites, in which the majority of the methylcytosines in the genome are found (Bird, 2002; Fazzari, 2004). Among these repetitive elements, LINE-1 retrotransposons comprise the largest component. They account for 20.1% of the entire human genome (Levy, 2007). Another example is the Alu-repetitive element, which makes up 13.1% of the human genome (Levy, 2007). Therefore, it is estimated that these two types of repetitive sequences together account for approximately one-third of DNA methylation and are suitable as surrogate markers for the genome-wide DNA methylation changes that are associated with multistep tumorigenesis in many types of cancers (Watanabe, 2010).

We used modified COBRALINE-1 and COBRAAlu techniques to conduct this study. These techniques were designed to detect 2 CpG dinucleotide sites, allowing us to determine not only the methylation level but also the methylation patterns of LINE-1 and Alu (Pobsook, 2011). At present, most quantitative methylation techniques, including pyrosequencing, can only measure the methylation level (usually from 3 CpG) and cannot distinguish specific LINE-1 and Alu methylation patterns. Moreover, overall methylation levels from this COBRA technique are proven to have linear correlations with the pyrosequencing technique (Jintaridh, 2010). Taken together, COBRALINE-1 and COBRAAlu are advanced and useful techniques for quantifying the methylation status of LINE-1 and Alu in genomic DNA.

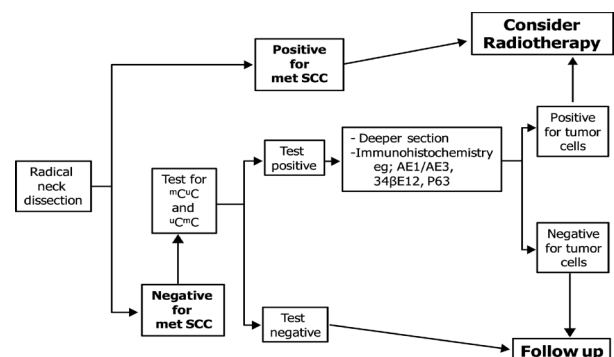
Various molecular markers have been proposed for investigating the presence of occult tumor metastases in histologically negative lymph nodes; these markers include P53 mutations (Ahrendt, 2002), K-ras mutations (Ahrendt, 2002), mRNAs of specific genes (Riethdorf, 2008) and the methylation of specific gene promoters (Harden, 2003). However, the targets for amplification of these markers exist as only 2 copies per cell and provided low efficacy when evaluating DNA from paraffin embedded tissues. In our study, we used interspersed repetitive sequences, including LINE-1 and Alu, as targets. The measurement of these markers has the advantage of increasing the sensitivity of detection of occult tumor metastases.

Our results confirmed that both LINE-1 and Alu demonstrated decreased methylation levels in the lymph nodes that contained metastatic disease compared to the cases without lymph node metastasis (N0). We also found that in the same group of patients with positive nodal status, negative nodes also demonstrated some evidence of hypomethylation, the level of which was intermediate between the cases with negative nodal status and the matched cases with true positive nodes. This phenomenon may be due to the effect of occult metastatic tumor, which is possibly not detected in routine pathological examination.

In this study, neither LINE-1 nor Alu methylation levels were correlated with the clinical stage or histological grade (data not shown), which was in agreement with the previous study (Smith, 2007). This finding may have been because our tumor samples were not retrieved using a microdissection procedure; therefore, the mixed cell types of the tumor and adjacent lymph node affected these results.

We also observed that the percentages of the  $^mC^uC$  and  $^uC^mC$  loci of LINE-1 were potentially suitable as supplement measures to detect lymph node metastasis because these measurements yielded the strongest differentiation power between the 3 groups of lymph nodes. By setting cut-off values for these two loci and applying them in combination, we can develop a test useful for distinguishing the lymph nodes that are positive for metastatic tumor. To obtain high sensitivity (as is yielded by FNA for screening metastatic lymph nodes), the test criteria of  $^mC^uC$  higher than 22.94% or  $^uC^mC$  lower than 23.98% is proposed. In contrast, if the test requires high specificity (i.e., to support a definitive diagnosis), the criteria of  $^mC^uC$  higher than 22.94% and  $^uC^mC$  lower than 23.98% should be applied. We do not recommend that this test be used for confirmation because, in our opinion, the histopathological examination should remain the gold standard for a definitive diagnosis. To integrate this test into clinical practice, an algorithm is proposed for the management approach in the routine radical neck dissection of lymph nodes (Figure 6).

In conclusion, our study clearly demonstrated the difference in the hypomethylation of both LINE-1 and Alu between the lymph nodes with metastatic HNSCC and the cases with negative nodes. We also found that the negative nodes from cases with metastatic nodal status demonstrated some tendency toward hypomethylation, which, in our opinion, implied occult metastatic tumor. Apart from confirming the occurrence of hypomethylation in HNSCC that has been reported in previous studies (Smith, 2007), our findings also suggest the potential use of this technology as an ancillary tool for detecting occult metastatic tumor in lymph node metastases; however, tumor cells are not detected by routine pathological



**Figure 6. Proposed Algorithm for the Diagnosis and Management of Radical Neck Dissection with Integration of  $^mC^uC$  and  $^uC^mC$  into the Approach.** \*met SCC, metastatic squamous cell carcinoma; IHC, immunohistochemistry. \*\*Test positive =  $^mC^uC$  higher than 22.94% and  $^uC^mC$  lower than 23.98%, Test negative =  $^mC^uC$  lower than 22.94% or  $^uC^mC$  higher than 23.98%.

examination. This technique may also be helpful for the diagnosis of metastatic diseases in fine-needle-aspiration cytology specimens. Despite a strong possibility for use in future clinical practice, these data are still post diagnosis evaluation. Further study in prediagnosis set up is warranted before this technique can be employed as a part of a laboratory investigation.

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