

RESEARCH ARTICLE

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LINE-1 and Alu Methylation in hrHPV-Associated Precancerous Cervical Samples

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Abstract

Objective: This study was conducted to determine global DNA methylation patterns in cervical cells cytologically identified as atypical squamous cells of unknown significance (ASCUS) with a normal, LSIL, or HSIL histopathological result. **Methods:** Methylation patterns of long interspersed nuclear elements (LINE-1) and short interspersed element (Alu) sequences were assessed using the combined bisulfite restriction analysis (COBRA) method in cervical samples with cytology-diagnosed cervical lesions. **Results:** In cervical precancerous lesions with hrHPV positive, the percentage of overall (^mC) and ^mC^mC LINE-1 methylation levels showed a stepwise increase from hrHPV positive normal to HSIL with significant differences (p<0.001). However, both methylation levels were significantly higher in hrHPV negative normal than in hrHPV positive normal (p<0.001). The overall (^mC) Alu methylation in hrHPV positive LSIL and HSIL was lower than in hrHPV positive normal, with a significant difference (p<0.05). Remarkably, the percentage of ^mC^mC and ^mC^uC of LINE-1 and Alu in three different hrHPV positive cervical lesions showed a stepwise decrease from hrHPV positive normal, LSIL and HSIL, respectively. Furthermore, receiver operating characteristic (ROC) curve analyses revealed that the LINE-1 ^mC and ^mC^mC patterns have high sensitivity and specificity for distinguishing HSIL from normal/LSIL in hrHPV positive cases at the appropriate cutoff levels. **Conclusion:** We have demonstrated the LINE-1 and Alu methylation data in normal and premalignant cervical epithelia. LINE-1 hypomethylation was found in hrHPV positive normal cells, with lower methylation levels associated with cancer features. In cytologically diagnosed Atypical Squamous Cells of Unknown Significance (ASCUS), the levels of ^mC and the ^mC^mC pattern could be utilized in concert with hrHPV detection to classify the ASCUS sample prior to colposcopy.

Keywords: Cervical cytology- hrHPV- SIL- Global methylation

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Introduction

Cervical cancer is the fourth most common cancer in women worldwide, however, it still has a high incidence and fatality rate in developing countries (Bray et al., 2018; Arbyn et al., 2020). Persistent hrHPV (high risk-human papillomavirus) infections caused cellular gene changes due to unregulated viral oncogene expression (Ganguly and Parihar, 2009; Gupta et al., 2018). In cervical cancer, hrHPV types 16 is discovered in more than 50% of cases, while HPV18 is detected in 20% of cases. Other hrHPV varieties, such as 31, 33, 45, 52, and 58, are present in about 30% of cervical cancer cases (Clifford et al., 2003). However, cancer develops in a small percentage of hrHPV

infected women (Melnikow et al., 1998).

Epigenetics alterations such as global DNA hypomethylation of high repetitive DNA sequences is a biological event that signals genomic instability and have been found in a variety of cancers including cervical cancer (Shuangshoti et al., 2007; Ehrlich, 2009). Chromosomal damage and instability were discovered in peripheral blood lymphocytes and cervical cells obtained from patients with Low grade SIL (squamous intraepithelial lesions, LSIL) and high grade SIL (HSIL) when compared to normal cells (Milošević-Djordjević et al., 2011; Cortés-Gutiérrez et al., 2013).

Previous studies have shown that the COBRA (combined bisulfite restriction analysis) method for

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detecting LINE-1 and Alu methylations can be utilized to distinguish cancer cells from normal cells (Shuangshoti et al., 2007; Puttipanyalears et al., 2013; Sirivanichsuntorn et al., 2013). LINE-1 and Alu are dispersed repetitive sequences along the whole genome. The methylation of both regions was utilized as representative areas for global methylation (Ushida et al., 2012; Li et al., 2014; Joyce et al., 2016). Furthermore, LINE-1 and Alu methylation patterns have been demonstrated to be useful indicators for distinguishing lesions in cancer multistep processes (Wangsri et al., 2012; Sirivanichsuntorn et al., 2013). This pattern was described as methylation and unmethylation statuses in two CpG sites as ${}^mC^mC$, ${}^mC^uC$, ${}^uC^mC$ and ${}^uC^uC$ (Pobsook et al., 2011).

The aims of this study were to find global DNA methylation patterns in cervical cells cytologically diagnosed as ASCUS (atypical squamous cells of unknown significance) with histopathological result as normal, LSIL, or HSIL. In addition, the existence of hrHPV types in cervical samples and different patterns of global DNA methylation, a sign of chromosomal instability, were studied.

Materials and Methods

Clinical specimens

Cervical samples were collected from left over cervical cells after routine hrHPVs testing by cobas®4800 (Roche diagnostic, IN, USA) at the Department of Microbiology, Faculty of Medicine, Chulalongkorn University, Bangkok, Thailand. All of the cases had a cytological diagnosis of ASCUS. Histopathological features of a colposcopic directed biopsy specimen were used to determine the definite diagnosis in this study and categorized as normal, LSIL (Cervical intraepithelial neoplasia (CIN) 1) and HSIL(CIN2,3). In total, 108 samples were collected including normal (n=60), LSIL (n=27) and HSIL (n=21). There is no statistical difference in age between all groups, as shown in Table 1. This study has been approved by the Institutional Review Board of the Faculty of Medicine, Chulalongkorn University (IRB no 148/60,COA no 389/2017).

hrHPV detection

HPV DNA was detected using the Cobas4800® HPV Test (Roche, Switzerland) based on real-time PCR (RT-PCR) for simultaneous detection of HPV16, HPV18, and 12 other high-risk HPVs (HPV31, -33, -35, -39, -45, -51, -52, -56, -58, -59, -66, and -68, as a pooled result). β -globin gene was used as a genomic DNA control to confirm the quality of samples. hrHPV was found in all LSIL and HSIL patients. We found hrHPV infection in 22 of the normal cases, while 38 were hrHPV negative. As a result, we divided the normal into hrHPV positive and hrHPV negative categories.

DNA extraction and bisulphite modification

Following the manufacturer's protocol, DNA was extracted from cervical cells using AllPrep DNA/RNA (Qiagen, Hilden, Germany). Extracted DNA samples were then bisulfite modified using the EZ kit Gold

Bisulfite Conversion Kit ((Zymo Research, CA, USA) in accordance with the manufacturer's instructions. When sodium bisulfite was added to deaminated cytosine residues, the unmethylated cytosine was changed to uracil, but the methylated cytosine remained unchanged.

COBRA of LINE-1 and Alu

We used the COBRA method to check for both LINE-1 and Alu sequence methylation at two CpG universal sites. This method used PCR amplification and restriction enzyme manipulation in the same way that previous research had done (Tiawech et al., 2014; Arayataweegool et al., 2019). Briefly, primers used for COBRA LINE-1 were forward 5'-CCGTAAGGGGTTAGGGAGTTTTT-3' and LINE-1 reverse 5'-RTAAAACCCCTCCRAACCAAATATAAA-3' and COBRA Alu were forward-5'-GGRGRGGTGGTTTARGTTTGTA-3' and Alu reverse 5'-TTTTTATATTTTTAATAAAAACRAAATTCACCA-3'. The annealing temperature for LINE-1 and Alu were 50°C and 53°C, respectively, for 40 cycles of PCR. Following PCR amplification, the LINE-1 (160 bp) amplicons were digested with 2 units of *TaqI* and *TasI* in NEB buffer 3 (New England Biolabs, Ontario, Canada), whereas the Alu amplicons (117 bp) were digested with *TaqI* in *TaqI* buffer (MBI Fermentas, Burlington, Canada). Both digestion reactions were kept at 65°C for the duration of the experiment. After that, the digested amplicons were electrophoresed on an 8% non-denaturing polyacrylamide gel and stained with SYBR green nucleic acid gel stain (Gelstar, Lonza, USA). Using a phosphorimager and Image Quant Software, the intensities of both COBRA PCR fragments were measured (Molecular Dynamics, GE Healthcare, Slough, UK). As a negative control, distilled water was used. To standardize inter-assay variation, DNA samples from HeLa cell lines were used as positive controls in each experiment. All of the experiments were carried out twice.

LINE-1 and Alu methylation analysis

The intensity of each band of the COBRALINE-1 and COBRAAlu amplicons was measured and calculated for five LINE-1 and Alu sequence patterns as follows: ${}^mC^mC$, ${}^mC^uC$, ${}^uC^mC$ and ${}^uC^uC$, respectively. The formulas for computing the LINE-1 and Alu methylation patterns have been detailed in previous publications (Tiawech et al., 2014; Arayataweegool et al., 2019).

Statistical Analysis

An independent sample t-test and Kruskal-Wallis tests were performed to determine the difference in LINE-1 and Alu patterns between and among groups, respectively. Receiver operating characteristic curve (ROC curve) was performed to assess the diagnostic accuracy of global methylation. All calculations were performed using the SigmaPlot 12.0 and SPSS statistics 22. The results were considered statistically significant when the p-value was <0.05.

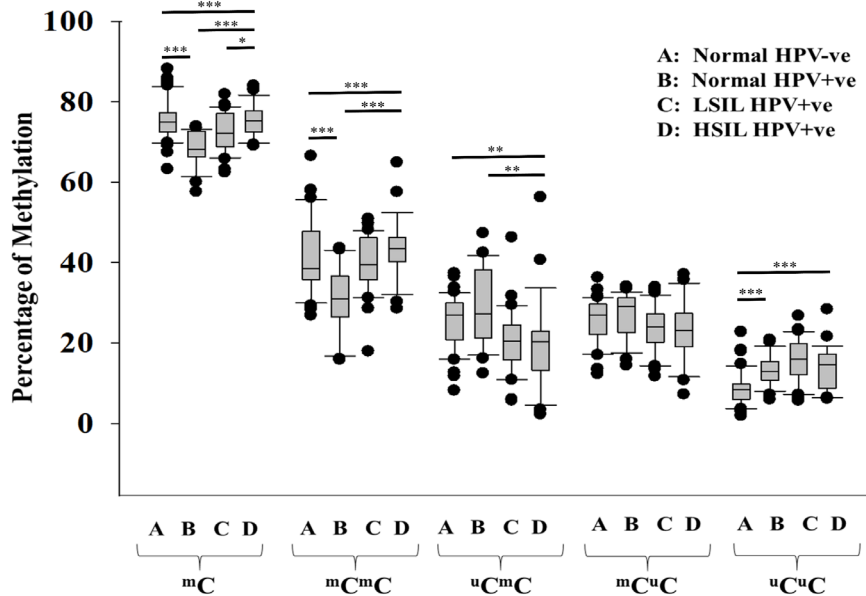


Figure 1. Comparisons of the Percentage of the Different Methylation Patterns of LINE 1 among Four Groups (* $p < 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$).

Results

Overall (mC) LINE-1 and Alu methylation

For LINE-1, the percentage of overall (mC) methylation among three different cervical lesions with hrHPV positive showed a stepwise increase from hrHPV positive normal to LSIL to HSIL with significant differences ($p < 0.001$) (Table 1, Figure 1). Interestingly, the methylation in hrHPV negative normal was higher than in hrHPV positive normal with a significant difference ($p < 0.001$). However, for Alu, the overall (mC) methylation in hrHPV negative normal was lower than in hrHPV positive normal with a significant difference ($p < 0.05$). When comparing HSIL to LSIL, the overall methylation of Alu was higher

($p = 0.098$).

Percentage of LINE-1 methylation in each pattern

Table 1 and Figure 1 show the various LINE-1 methylation patterns. With significant differences ($p < 0.001$), the percentage of $mCmC$ among three different cervical lesions with hrHPV positive showed a stepwise increase from hrHPV positive normal to LSIL to HSIL. The $mCmC$ pattern in hrHPV negative normals was significantly higher than in hrHPV positive normals ($p < 0.001$). However, among three different cervical lesions with hrHPV positive, the percentage of $uCmC$ and $mCuC$ showed a stepwise decrease from hrHPV positive normal to LSIL to HSIL. hrHPV negative normal had

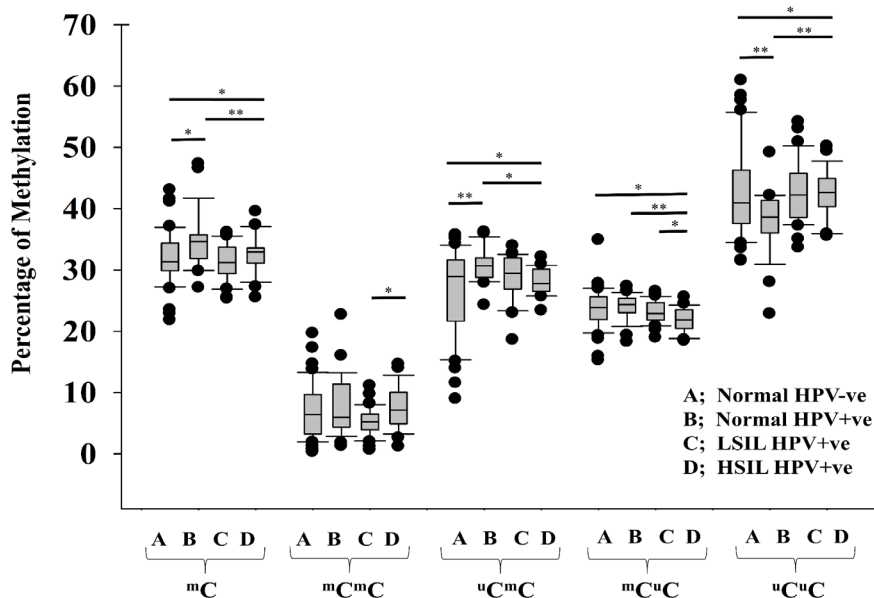


Figure 2. Comparisons of the Percentage of the Different Methylation Patterns of Alu among Four Groups (* $p < 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$).

Table 1. LINE-1 and Alu Methylation Level in Cervical Epithelial Cells of Various Lesions

Cytology		Normal	LSIL	Normal+LSIL	HSIL		p-value	(95% CI)			
hrHPV results		Negative	Positive	Positive	Positive						
N		38	22	27	49	21	A vs B vs C vs D	(B+C) vs D	B vs C vs D	A vs B	C vs D
Age (median (range))		46 (32-57)	46 (32-71)	46 (33-58)	46 (32-71)	42 (33-55)	A vs B vs C vs D	(B+C) vs D	B vs C vs D	A vs B	C vs D
		A	B	C	B+C	D					
LINE-1 methylation (average(%)±S.D.)	^m C	75.60±5.38	68.32±4.65	72.56±5.16	70.66±5.33	75.41±4.11	<0.001	<0.001	<0.001	<0.001	0.045
	^m C ^m C	40.84±9.18	30.94±8.53	39.87±7.59	35.86±9.12	43.58±8.07	<0.001	0.001	<0.001	<0.001	0.109
	^u C ^m C	25.02±6.88	29.18±9.83	20.73±8.38	24.52±9.92	20.03±12.35	0.003	0.112	0.005	0.059	0.819
	^m C ^u C	25.48±5.85	26.71±6.00	23.50±5.90	24.94±6.10	22.75±7.89	0.131	0.212	0.112	0.441	0.707
	^u C ^u C	8.66±4.44	13.17±4.03	15.90±5.39	14.68±4.97	13.64±5.59	0.001	0.442	0.134	<0.001	0.161
Alu methylation (average(%)±S.D.)	^m C	31.99±4.62	34.80±4.81	31.26±3.07	32.85±4.29	32.51±3.34	0.023	0.743	0.007	0.029	0.188
	^m C ^m C	6.94±4.53	7.51±5.12	5.31±2.34	6.29±3.95	7.54±3.61	0.176	0.22	0.065	0.655	0.013
	^u C ^m C	26.44±7.11	30.68±2.80	28.83±3.61	29.66±3.37	28.11±2.19	0.014	0.058	0.018	0.01	0.429
	^m C ^u C	23.67±3.43	23.90±2.25	23.08±1.88	23.45±2.07	21.81±1.99	0.038	0.003	0.005	0.778	0.029
	^u C ^u C	42.96±7.77	37.92±5.42	42.78±5.09	40.59±5.73	42.53±4.05	0.013	0.165	0.002	0.009	0.456

lower ^uC^mC, ^mC^uC, and ^uC^uC patterns than hrHPV positive normal.

Percentage of Alu methylation in each pattern

For Alu methylation, the different Alu methylation patterns were shown in Table 1 and Figure 2. The percentage of ^mC^mC pattern was similar level in hrHPV positive normal when comparing to hrHPV negative normal and similar trend was found when compared HSIL to LSIL. The percentage of ^uC^mC and a ^mC^uC among three different cervical lesions with hrHPV positive showed a stepwise decrease from hrHPV positive normal to LSIL to HSIL. When compared to hrHPV positive normal, the ^uC^mC and ^mC^uC methylation levels in hrHPV negative normal were slightly lower.

LINE-1 methylation is used to distinguish normal/LSIL hrHPV lesions from HSIL hrHPV lesions

Normal/LSIL lesions have a distinct clinical treatment

strategy than HSIL lesions. For this reason, ROC curves were utilized to analyze the sensitivity and specificity of LINE-1 methylation with the suitable cutoff. The ^mC and ^mC^mC patterns of LINE-1 sequences were chosen as the probable patterns to use. Figure 3 and Table S1 demonstrated an area under the area under the ROC curve (AUC) value of ^mC = 0.755 when the cut off level was more than or equal to 70.69%. The outcome of this was that the sensitivity was 85.71%, while the specificity was just 55.10% to diagnose HSIL. Whereas, when the cut off level of ^mC^mC was greater than or equal to 40.55%, the AUC value = 0.716. This resulted in a sensitivity of 76.19% and a specificity of 69.39% for HSIL diagnosis.

Discussion

One of the most prevalent epigenetic alterations in the mammalian genome, DNA methylation, has been associated to tumorigenesis (Kitkumthorn and

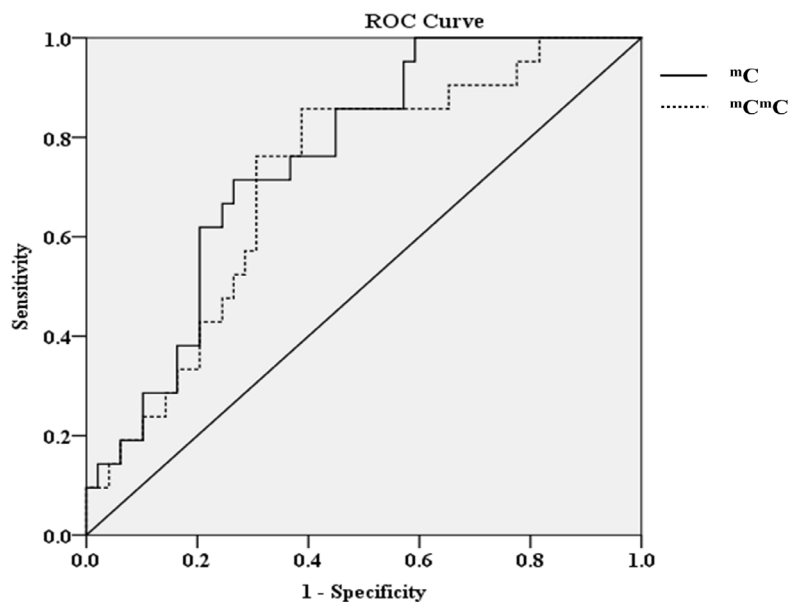


Figure 3. Sensitivity and Specificity of ^mC and ^mC^mC LINE-1 Methylation Patterns to Differentiate between hr-HPV Positive normal/LSIL and HSIL Lesions. AUC of ^mC and ^mC^mC were 0.755 and 0.716, respectively.

Mutirangura, 2011). These alterations have a shared influence in widespread repetitive DNA sequences, which contain the majority of the methylcytosines in the genome, and are known as global methylation (Bird, 2002; Fazzari and Greally, 2004). LINE-1 is the most abundant of these repeating components, accounting for around 20.1 % of the whole human genome, whereas Alu elements account for 13.1 % (Levy et al., 2007). Both are suitable as surrogate indicators for global methylation to motoring the cancer association (Kitkumthorn et al., 2012).

The COBRALINE-1 and COBRAAlu techniques were used in this work to identify global DNA methylation, the results showed progressive hypomethylation from hrHPV positive normal cervical cells to hrHPV positive abnormal cervical samples. Our findings were consistent with earlier pyrosequencing studies that demonstrated HSIL/CIN2/3 had greater global DNA methylation values than LSIL/CIN1 (Thumbovorn et al., 2022). Among various different methods to detect DNA methylation (Karbalaie Niya et al., 2019), the COBRA approach is advantageous in this regard because it is simpler and less expensive. Furthermore, these approaches were intended to detect two CpG dinucleotide sites, allowing us to determine not only the quantity of methylation but also the methylation patterns of LINE-1 and Alu (Pobsook et al., 2011).

Several gene-specific methylation biomarkers (Chujan et al., 2014; Oranratanaphan et al., 2020; El Aliani et al., 2021) have been proposed for differentiated cytological cervical cancer. However, because the markers' amplification targets only come in two copies, we used numerous copies of repetitive sequences like LINE-1 and Alu as targets. The benefit of measuring these indicators is that it improves detection sensitivity.

The outcomes of this study indicated overall LINE-1 increased methylation levels in hrHPV positive case from normal to LSIL and HSIL similar trend to the study of Flatley et al., (2009) studies but different to the studies of Thumbovorn R et al. and Shuangshote et al., (2007); Thumbovorn et al., (2022). In addition, hrHPV-positive normals had a higher overall Alu methylation level than hrHPV-negative normals, according to the study. In contrast, Sen S. et al. reported that Alu hypomethylation was seen in HPV16 infected non-malignant cervical samples when compared to HPV negative normal control samples (Sen et al., 2017). Controversies may be due to the diverse types and methods of tissue harvest. Surprisingly our data indicated higher degree of LINE-1 methylation in hrHPV negative normal samples compared to hrHPV positive normal samples. The difference in hypomethylation levels between the two groups showed that the infection with hrHPV led to alterations in the cellular environment.

In addition, we found that the percentages of ^mC and ^mC^mC of LINE-1 methylation could be utilized to differentiate between normal/LSIL and HSIL hrHPV positive cytological samples. In cases where cytological assessment reveals ASCUS with the presence of hrHPV, these techniques are useful for screening cytological samples for hrHPV positive with a high degree of sensitivity. However, the study's limitations may cause some concern. The cytological sample contains some

normal cells, which may interfere with the results. Furthermore, our study had a small sample size. As a result, further research should be conducted with a large sample size to confirm its existence.

We do not really advocate this test for confirmation because histological investigation should be the gold standard for a confirmed diagnosis, in our opinion. However, in conjunction with hrHPV detection, these assays could be used to classify the ASCUS sample prior to colposcopy.

Author Contribution Statement

NK and AC designed the study, analyzed, and interpreted data. SB, SC and AC performed the experiments. AC drafted manuscript. NK reviewed and edited the manuscript. All authors read and approved the final manuscript.

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Ethical Declaration

This study has been approved by the Institutional Review Board of the Faculty of Medicine, Chulalongkorn University (IRB no 148/60,COA no 389/2017).

Conflict of Interest

The authors, hereby, declare no conflicts of interest.

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