

Detection of Global DNA Methylation in Cervical Intraepithelial Neoplasia and Cancerous Lesions by Pyrosequencing and Enzyme-Linked Immunosorbent Assays

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Abstract

Background: Cervical cancer is one of the most significant cancer found in women worldwide especially in developing countries. Previous reports showed that global DNA hypomethylation was correlated with various types of cancer including cervical cancer. **Methods:** Long interspersed nuclear element-1 (LINE1) pyrosequencing and Enzyme linked-immunosorbent assay (ELISA) assays were used for detection of global DNA methylation. The ELISA results were compared to bisulfite LINE1 pyrosequencing assay. **Results:** Different cervical cancer cell lines (CaSki, SiHa, HeLa, ME180, MS751, C33A) showed low global methylation percentage when compared to normal white blood cells by ELISA assay (1.47%-5.09% vs 8.20%, respectively) and by LINE1 pyrosequencing (20%-45% vs 62%, respectively). Global DNA methylation levels in cervical cancer samples were lower than precancerous lesions (Normal-CIN3) by LINE1 pyrosequencing (mean, 48.8% vs 56.9%, respectively, $p < 0.05$) and ELISA assay (mean, 3.03% vs 3.85%, respectively, $p < 0.05$). **Conclusion:** Global DNA hypomethylation was predominantly found in cervical cancer samples detected by ELISA and LINE1 pyrosequencing assays and could be used as triage tests in cervical cancer screening. ELISA assay is a suitable method for detection of global DNA methylation in large population; however, it should be further evaluated in a large clinical samples in order to be used as screening method.

Keywords: Global DNA methylation- enzyme linked immunosorbent assay- Long interspersed nuclear element-1

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Introduction

Cervical cancer is one of the most common cancer found in women worldwide, approximately >500,000 cervical cancer cases were diagnosed annually and nearly 50% death (Torre et al., 2017). In low- and middle-income countries, cervical cancer is the second most frequently anogenital cancer found in women after breast cancer (Woodman et al., 2007; Torre et al., 2017).

Persistent infection with high risk or oncogenic human papillomavirus (HPV) types is accepted as a necessary cause of cervical cancer. Among high risk-HPV types, HPV 16 and HPV 18 cause >70% of cervical cancers worldwide (Li et al., 2011). HPV 16 is detected more than 50% in squamous cell carcinoma and HPV18 is mostly found in adenocarcinomas (Motoyama et al., 2004; Gnanamony et al., 2007). HPV 58 and 52 are mostly found in eastern Asian countries after HPV16 and 18 (Chan et al., 1999; Hwang, 1999; Chen et al., 2006; Inoue et al., 2006; Sukasem et al., 2011; Aromseree et al., 2014). However, the majority of HPV infected women has spontaneous regression, minor of them develop cancer (Melnikow et al.,

1998). Epigenetics changes are involved in carcinogenesis, one of these changes is global DNA hypomethylation of high repetitive DNA sequences such as long interspersed nuclear elements or LINE and Alu sequences (Ehrlich, 2009). Global DNA hypomethylation was found in most cancer types such as breast cancer (Wu et al., 2011) prostate cancer (Yegnasubramanian et al., 2008) bladder cancer (Choi et al., 2009) liver cancer (Takai et al., 2000) chronic myeloid leukemia (Roman-Gomez et al., 2008) and cervical cancer (Kim et al., 1994; Missaoui et al., 2010; Sen et al., 2017) that may have potential to be used as biomarker for screening of cancer including cancer of anogenital tracts.

Previous reports showed global DNA methylation in cervical cancer samples by detecting *LINE1* gene methylation using combined bisulfite restriction analysis (COBRA) polymerase chain reaction and found association between *LINE1* hypomethylation and cancer stage (Shuangshoti et al., 2007). Other group performed immunohistochemistry using antibody to 5-methylcytosine in formaline fixed paraffin embedded tissue (FFPE) and using high-performance capillary

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electrophoresis (HPCE), they found that global DNA hypomethylation was associated with cervical cancer (Missaoui et al., 2010).

Various assays have been used to detect global DNA methylation by detecting LINE1, Alu, Satellite-alpha and Satellite-2 regions using molecular assays such as real time methylation specific PCR, bisulfite sequencing PCR, combined bisulfite restriction analysis (COBRA) and pyrosequencing (Li et al., 2014; Kurdyukov and Bullock, 2016). However, these techniques need bisulfite treatment, require expensive equipments and are not suitable in low income countries for screening in general population. Therefore, we aimed to use enzyme linked immunosorbent assay (ELISA) that is more suitable to be used in low resource countries and high-throughout screening assay for detection of global methylation. DNA extracted from cervical cells samples with different lesions severity were employed. Results of ELISA assay were compared to bisulfite LINE1 pyrosequencing.

Materials and Methods

Clinical samples and cell lines

Left over cervical cells were collected from routine Pap smears at the Department of Gynecological Outpatient, King Chulalongkorn Memorial Hospital and the Department of Microbiology, Faculty of Medicine, Chulalongkorn University, Bangkok, Thailand. DNA was extracted from cervical cells by AllPrep DNA/RNA (Qiagen, Hilden, Germany). (In total, 280 samples were collected including normal (n=36), cervical intraepithelial neoplasia 1 (CIN1) (n=115), CIN2-3 (n=39) and squamous cell carcinoma (SCC) (n=90). Caski (500-600 copies of HPV16), SiHa (1-2 copies of HPV16), HeLa (HPV18 positive), ME180 (HPV39), MS751 (HPV45) and C33A (HPV negative) cell lines were used as cancerous cells control. White blood cells collected from a healthy donor was used as normal control. Buffy coat was removed from EDTA blood and used for DNA extraction using AllPrep DNA/RNA (Qiagen, Hilden, Germany). This study has been approved (COA No. 087/2016) by the Institutional Review Board of the Faculty of Medicine, Chulalongkorn University.

Bisulfite conversion of DNA extracted from clinical samples and LINE1 methylation analysis by pyrosequencing

DNA samples were converted using the EZ kit Gold Bisulfite Conversion Kit (Zymo Research) according to the manufacturer's instruction. The extracted DNA (100-1000 ng) (maximum 20µL) was used in bisulfite modification reaction. DNA extracted from human cervical cell lines (CaSki, SiHa, ME180, MS751 and C33A) were used as cancerous cells control and DNA extracted from normal white blood cell was used as normal control for amplification and pyrosequencing.

The sequences of the LINE1 forward and reverse primers were FW: 5'-TTTTGAGTTAGGTGTGTGGGATATA-3' and RV: Biotin-5'-AAAATCAAAAATTC CTTTC-3' (150 bp). Sequencing primer and sequence to analyze were 5'-AGTTAGGT GTGGGATATAGT-3 and TGGTGYGTGTTTTTAAGTYGGTTTGAAAAGYA

TA, respectively (Delaney et al., 2015). Bisulfite modified DNA samples were used for PCR amplification as follows: 13.6 µL DNase/RNase free water, 1X PCR buffer, 2.5mM MgCl₂, 250µM dNTP, 12.5pM of each forward and reverse primers, 1 Unit DNA polymerase (HotStart HiFidelity Polymerase, Affymetrix, USA). The PCR conditions were as follows: initial denaturing at 95°C for 10 minutes, followed by 50 cycles of 95°C for 30 seconds, 50°C for 1 minute and 72°C for 30 seconds and final extension at 72°C for 5 minutes. The PCR products were detected by 1.5% agarose gel electrophoresis. Prior to pyrosequencing, 20µL of biotin labeled amplified products were mixed with beads, washed with 70% ethanol, denatured, washed and mixed with 0.4µM of sequencing primers and loaded into the PyroMark™ Q96 machine (Qiagen, Hilden, Germany).

Quantification of global DNA methylation by ELISA-based assay

ELISA was performed according to instruction manual (5-mC DNA ELISA Kit, Zymo Research)1. Briefly, 100 ng of DNA samples were denatured and placed on ice immediately for 10 minutes and subsequently applied to an ELISA well strip. After DNA-surface binding, primary antibody against 5-methyl cytosine and secondary antibody were applied in an ELISA strip well. After color development, the absorbance was read at 405 nm using a microplate reader (Perkin elmer Victor). The absolute quantity of global DNA methylation was determined by subtracting the sample OD from the negative control and calculate the percentage of methylation using equation achieved from a standard curve, 0%, 5%, 10%, 25%, 50%, 75% and 100%, which was obtained from positive control DNA with known 5-methyl cytosine concentration, running on the same plate in duplicate.

Statistical analysis

The Kruskal–Wallis test was used to analyze the differences of the mean methylation values among groups of specimens. Receiver operating characteristic (ROC) curve was used to analyze the sensitivity and specificity to diagnose lesion severity with cancerous lesion. P-value less than 0.05 was considered statistically significant.

Results

Methylation levels in cervical cancer cell lines

The optimal time and wavelength for absorbance measurement by ELISA assay were 40 min at 405 nm. The results of global methylation percentage by ELISA found in various cervical cancer cell lines infected with different HPV types were as followed, 3.72% (Caski), 1.74% (SiHa), 2.42% (HeLa), 4.79% (ME189), 5.09% (MS751) and 1.47% (C33A) while DNA extracted from normal white blood cells showed 8.20%. Bisulfite LINE1 pyrosequencing results were 45% of Caski, 35% of SiHa, 20% of C33A and 62% of normal white blood cells. Both assays showed global DNA hypomethylation in cervical cancer cell lines when compared to normal white blood cells.

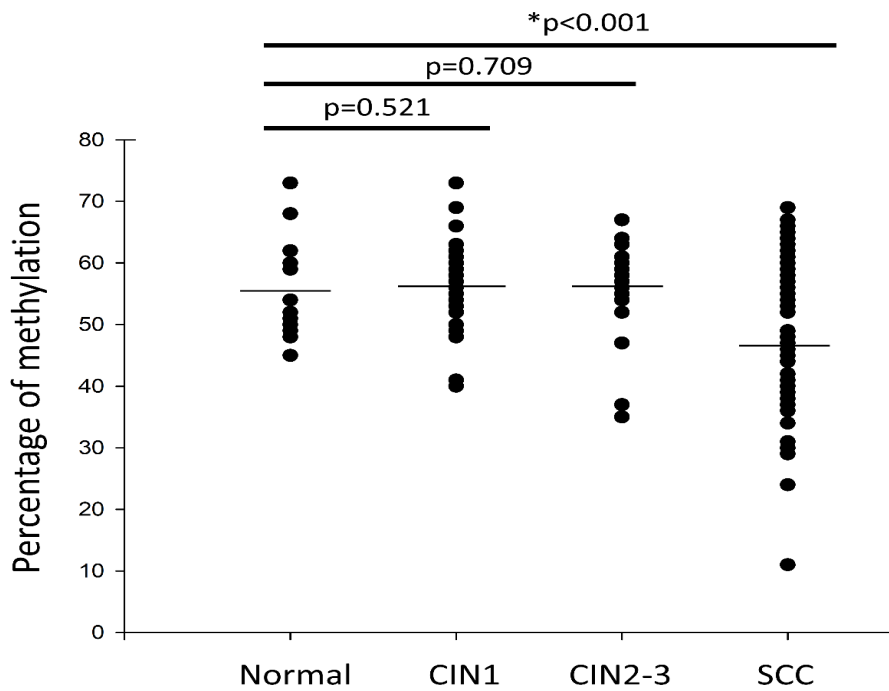


Figure 1. Methylation Levels in 167 Cervical Samples with Different Lesions Severity (* $p < 0.05$ (Kruskal wallis)).

Methylation levels in clinical samples

167 of 280 cervical samples were available for global DNA methylation by pyrosequencing and means of methylation were as followed; 55.6% of normal, 56.8% of CIN1, 57.3% of CIN2-3 and 48.8% of cervical cancer, there was a significant difference between cervical cancer samples and precancerous samples (normal-CIN3) ($p < 0.05$) (Figure 1). 257 of 280 samples were used for global DNA methylation detection by ELISA assay, however, samples with optical density (OD) less than 0.1 were excluded. Ninety-seven cervical samples were included for further methylation analysis and methylation means were as followed, 4.50% of normal, 3.83 of CIN1, 2.83% of CIN2-3 and 3.03% of cervical cancer, there was a significant difference between cervical cancer samples and precancerous

samples (normal-CIN3) ($p < 0.05$) (Figure 2). Global DNA methylation level detected by ELISA assay was proportional to LINE1 pyrosequencing assay ($N = 58$, $r = 0.442$, Figure 3).

Sensitivity and specificity of ELISA and LINE1 pyrosequencing assays for diagnosis of cervical cancerous lesions

We further analyzed whether the methylation level of a global DNA methylation by either bisulfite LINE1 pyrosequencing and ELISA assay could be used to predict cervical lesion severity. Receiver operating characteristic (ROC) curve was used to calculate the sensitivity and specificity at different methylation percentages as a cut off point to differentiate between precancerous and cancerous lesions. Figure 4 and 5 showed ROC curve and area under

Table 1. Sensitivity and Specificity of Global DNA Methylation to Differentiate between Precancerous (normal, CIN1, CIN2-3) and Cancerous Lesions by Bisulfite LINE1

LINE1 pyrosequencing.								
Cut off	30%		35%		40%		45%	
	Sensitivity	Specificity	Sensitivity	Specificity	Sensitivity	Specificity	Sensitivity	Specificity
%	4.76	100	9.52	100	23.81	97.59	36.9	95.18
Cut off	50%		55%		60%		65%	
	Sensitivity	Specificity	Sensitivity	Specificity	Sensitivity	Specificity	Sensitivity	Specificity
%	50	79.52	67.86	61.45	85.71	24.1	96.43	9.63
ELISA								
Cut off	1.50%		2.00%		2.50%		3.00%	
	Sensitivity	Specificity	Sensitivity	Specificity	Sensitivity	Specificity	Sensitivity	Specificity
%	17.86	91.3	28.57	78.26	42.86	60.87	57.14	56.62
Cut off	3.50%		4.00%		4.50%		5.00%	
	Sensitivity	Specificity	Sensitivity	Specificity	Sensitivity	Specificity	Sensitivity	Specificity
%	64.29	50.72	75	42.03	82.14	39.13	89.29	31.88

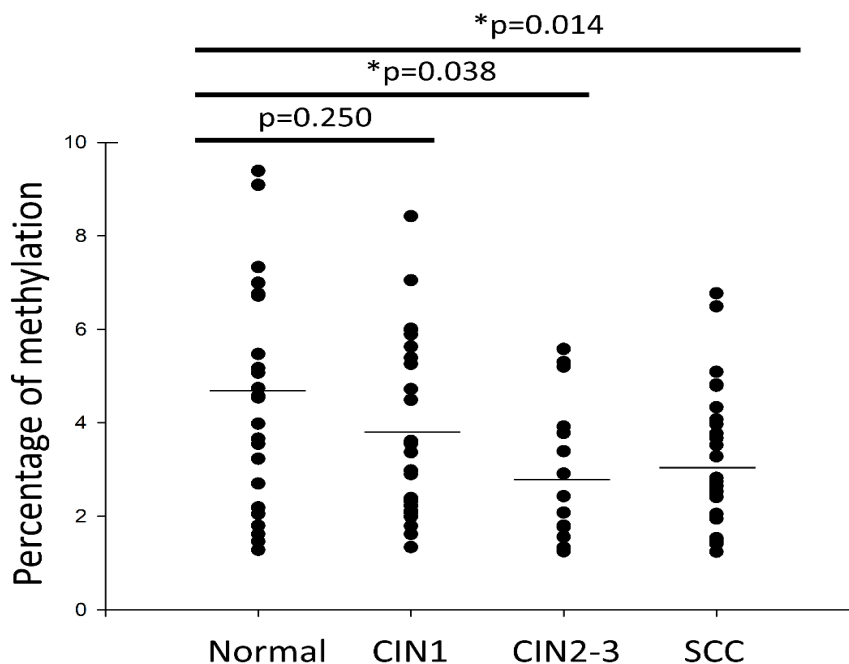


Figure 2. Methylation Levels in Ninety-Seven Cervical Samples with Different Lesions Severity Detected by ELISA assay (*=p<0.05 (Kruskal wallis)).

curve (AUC) of LINE1 pyrosequencing and ELISA (AUC=0.72 and 0.60, respectively). At cut off points, 30%-45% of LINE1 pyrosequencing and 1.5%-2.0% of ELISA, revealed high specificity but low sensitivity as shown in Table 1 , respectively.

Discussion

It has been shown that aberrant DNA methylation such as hypomethylation of human genome is associated

with various types of cancer (Ehrlich, 2009). Various qualitative assays such as direct sequencing of PCR products or TA cloning of PCR products and quantitative assay such as pyrosequencing need bisulfite treatment to convert unmethylated cytosine to uracil but not 5-methylcytosine (Kurdyukov and Bullock, 2016). ELISA based assay is the rough estimation of global DNA methylation, however, it is a quick and easy to perform assay and DNA can be directly detected without bisulfite treatment step. Furthermore, this assay is suitable for

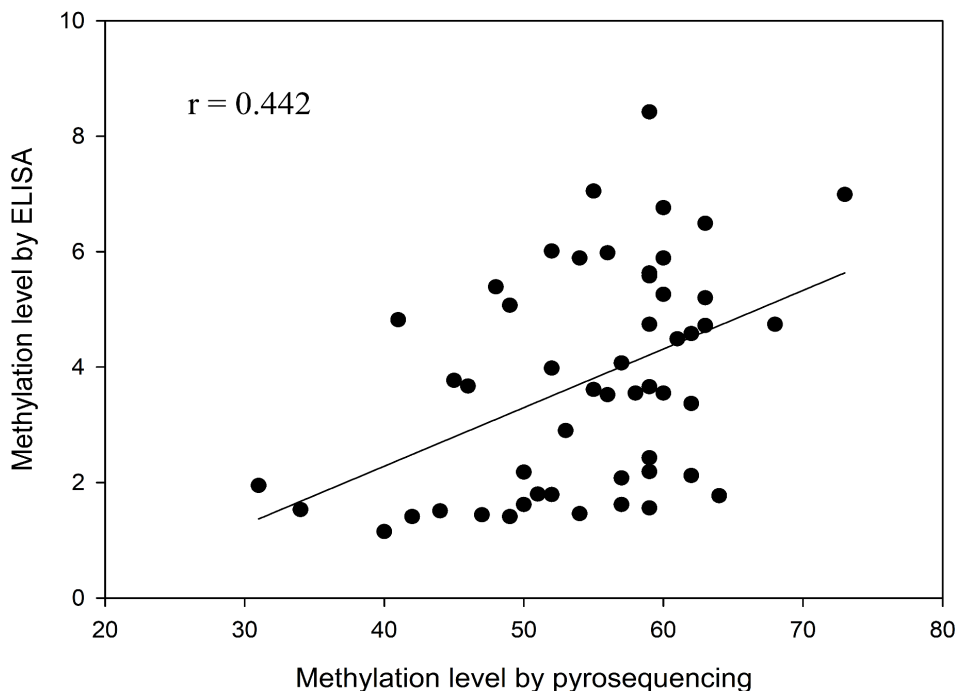


Figure 3. Correlation of Bisulfite LINE1 Pyrosequencing and ELISA Assays (N=58).

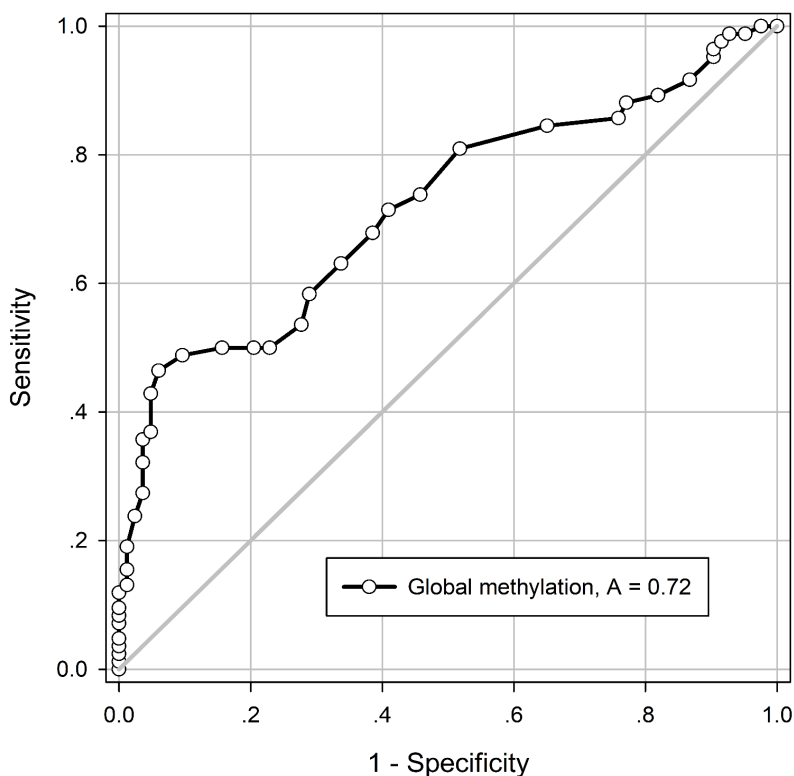


Figure 4. Receiver Operating Characteristic (ROC) Curve Shows the Sensitivity and Specificity of Methylation Levels to Differentiate between Precancerous (Normal,CIN1,CIN2-3) and Cancerous Lesions by Bisulfite LINE1 Pyrosequencing

high-throughput analysis of clinical samples especially in low resource countries because of its cost effectively (Kurdyukov and Bullock, 2016).

The present study employed bisulfite LINE1 pyrosequencing assay and ELISA based assay for analysis of global DNA methylation, the results indicated

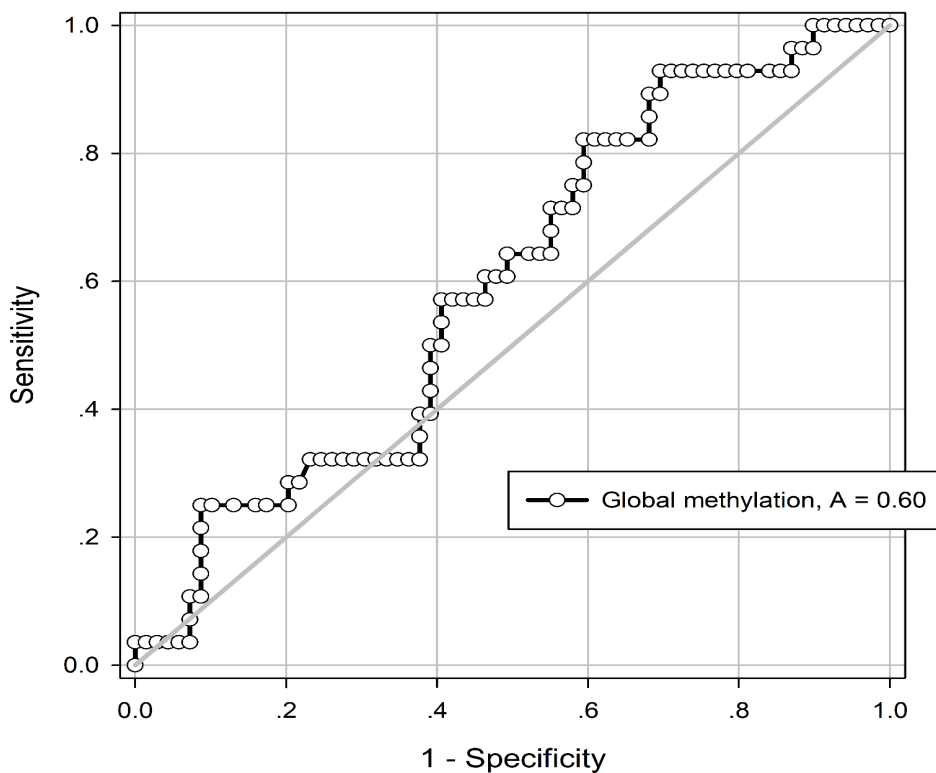


Figure 5. Receiver Operating Characteristic (ROC) Curve Shows the Sensitivity and Specificity of Methylation Levels to Differentiate between Precancerous (Normal,CIN1,CIN2-3) and Cancerous Lesions by ELISA Assay.

progressive global DNA hypomethylation from normal cervical cells to cervical cancer samples by both assays. Our results were in accordant to previous studies in various cancer types (Flatley et al., 2009; Hou et al., 2010; Akers et al., 2014). Akers et al. detected LINE1 methylation in ovarian cancer using bisulfite pyrosequencing and reported hypomethylation in epithelial ovarian cancer (57.1%) when compared to normal ovarian surface epithelia (72.5%) (Akers et al., 2014). Fotouhi et al reported LINE1 hypomethylation in small intestinal neuroendocrine tumors (65%) compared to normal cells (75%) by pyrosequencing and by ELISA assay, mean of methylation index in cancer samples was 1.1 when compared to methylation index 1.2 found in normal cells (Fotouhi et al., 2014). Foy et al found LINE1 hypomethylation in oral cancer compared non cancerous lesions (Foy et al., 2015). LINE1 hypomethylation was found in various types of cancer such as bladder, colon, pancreas, prostate and stomach cancers (Nüsgen et al., 2015). The study of global methylation in cervical cancer was reported using high-performance capillary electrophoresis (HPCE) assay, they reported hypomethylation in cancer cells that was 2.81 when compared to normal, low grade lesions and high grade lesions that were 3.26, 3.37 and 3.22, respectively, that suggested progressive hypomethylation (Missaoui et al., 2010). The other group reported progressive LINE1 hypomethylation level from normal epithelium to squamous cell carcinoma using combined bisulfite restriction analysis (COBRALINE1) PCR (Shuangshoti et al., 2007). The study using tritium-labeled methyl groups to incorporate into genomic DNA to detect global DNA methylation reported that most of cervical cancer samples had global DNA hypomethylation when compared to precancerous lesions (Flatley et al., 2009).

This study has some limitations, HPV DNA detection and typing was not performed in the present study, therefore, correlation between HPV status and global DNA hypomethylation could not be analysed. One study reported that global DNA hypomethylation was mostly found in HPV16 infected cervical cancer samples when compared to HPV negative normal control. Moreover, it was reported that HPV16 episomal form was correlated with global DNA hypomethylation than HPV16 integration form (Sen et al., 2017). However, one study in head and neck cancer showed contrary results, they reported LINE-1 hypomethylation was mostly found in HPV-negative than in HPV-positive cancer samples (Richards et al., 2009; van Kempen et al., 2014). In addition, LINE-1 hypomethylation was correlated with genomic instability (Richards et al., 2009). The DNA needed in ELISA reaction was 100 ng, nevertheless, input DNA used was less than 100 ng, therefore more than half of samples were excluded for further data analysis due to very low optical density (<0.1) that similar to negative control. In addition, the quantification of global DNA methylation level by ELISA assay (<10%) was particularly lower than results of LINE1 pyrosequencing assay. However, previous study used the same ELISA kit reported the similar percentage of global DNA methylation (<10%) compared to LINE1 methylation (Fa et al., 2016).

Signal amplification using horseradish peroxidase (HRP) enzyme system may be the reason of low global DNA methylation level compared to target amplification assay of LINE1 bisulfite pyrosequencing.

In conclusion, global DNA hypomethylation quantified by ELISA assay was at least correlated with quantitative bisulfite LINE1 pyrosequencing in term of progressive global DNA hypomethylation. ELISA based assay should be further developed and evaluated in large clinical samples size in order to obtain suitable cut off point and to be used as triage test in cervical cancer screening to differentiate women who are at high risk to develop cervical cancer quickly from low risk women.

Author Contribution Statement

AC designed the experiments and was a major contributor to writing the manuscript. RT performed the experiments. PB supervised the project and reviewed the manuscript.

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Data Availability Statement

Data supporting the findings of the present study are available within the article. Raw data that support the findings of the present study are available from the corresponding author, upon reasonable request.

Ethical approval

This study has been approved (COA No. 087/2016) by the Institutional Review Board of the Faculty of Medicine, Chulalongkorn University. This article was not a part of student thesis.

Conflict of Interest

The authors declare that there is no conflict of interest.

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