Detection of global methylation in cervical intraepithelial neoplasia and cancer by enzyme linked immunosorbent assay and LINE1 methylation.

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Abstract

Introduction; Cervical cancer is the second most cancer found in women worldwide especially in developing countries including Thailand. Previous reports showed that global DNA hypomethylation is found in many types of cancer including cervical cancer. We aimed to develop enzyme linked immuno sorbent assay (ELISA) for detection of global DNA methylation in order to screening of cervical cancer in low resource countries.

Materials and methods; ELISA assay was developed and the results obtained from ELISA assay were compared to bisulfite LINE1 methylation by pyrosequencing. DNA extracted from cervical cells with different lesions severity were used.

Results; By ELISA assay, the percentage of global DNA methylation in cervical cancer cell lines were 3.72 (Caski), 1.74 (SiHa), 2.42 (HeLa), 4.79 (ME189), 5.09 (MS751), 1.47 (C33A) and 8.20 of white blood cells collected from normal population. Bisulfite LINE1 pyrosequencing were 45% of Caski, 35% of SiHa and 62% of normal white blood cells. Mean of global DNA methylation by pyrosequencing in 161 cervical samples were as followed; 55.6% of normal, 56.8% of CIN1, 57.3% of CIN2-3 and 48.8% of cervical cancer, there was significant difference among groups ($p<0.05$). By ELISA assay in 100 cervical samples, 4.50% of Normal, 3.83 of CIN1, 2.83% of CIN2-3 and 3.03% of cervical cancer, there was significant difference among groups ($p<0.05$).

Discussion; Global DNA hypomethylation was significantly found in cervical cancer compared to normal cervical cells. Problem of ELISA assay is the binding affinity of DNA on ELISA plate leading to the low percentage of global DNA methylation when compared to LINE1 pyrosequencing assay. ELISA assay must be further developed and evaluated in clinical samples for reliable results in order to be used as screening method.

Keywords; Global DNA methylation, enzyme linked immuno sorbent assay, LINE1, pyrosequencing

1. Introduction

Cervical cancer is the second most common cancer found in women in low income countries. [1] Approximately 500,000 cervical cancer cases were diagnosed annually and most of them were found in women in developing countries including Thailand. In Thailand, more than 6,000 cervical cancer cases were found and half of them die each year [2].

Human papillomavirus (HPV) is accepted as a necessary cause of cervical cancer and among high risk HPV, HPV type 16 and HPV type 18 cause 70% of cervical cancers and precancerous lesions . HPV16 is detected more than 50% in squamous cell carcinoma and HPV18 is mostly found in adenocarcinomas [3, 4]. HPV58 and 52 are mostly found in eastern Asian countries after HPV16 and 18 [5-9].

The majority of HPV infected women has spontaneous regression, minor of them develop cancer. Epigenetics changes are involved in carcinogenesis such as hypermethylation of host tumor suppressor gene promoter. Global hypomethylation of high repetitive DNA sequences such as long interspersed nuclear elements or LINE and Alu sequences were reported in many cancer samples such as breast cancer [10] prostate cancer [11] bladder cancer [12] liver cancer [13] chronic myeloid leukemia [14] and cervical cancer [15] 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 [16] using combined bisulfite restriction analysis (COBRA) and found association between LINE1 hypomethylation and cancer stage. Other group performed immunohistochemistry using antibody to 5-methylacytosine in formaline fixed paraffin embedded tissue (FFPE) and using high-performance capillary electrophoresis (HPCE), they found global DNA hypomethylation is associated with cervical cancer [15].

Various assays have been used to detect global DNA methylation by detecting LINE1, Alu, Satellite-alpha and Satellite-2 regions [17] using molecular assays such as real time methylation specific PCR, bisulfite sequencing PCR, combined bisulfite restriction analysis (COBRA) and pyrosequencing. However, these techniques need expensive equipments and are not suitable in low income countries for screening in general population. We aimed to develope Enzyme Linked Immunosortbent (ELISA) assay that is more suitable to be used in low resource countries and for high throughout screening. DNA extracted from

cervical cells samples with different lesions severity will be employed. Results of ELISA assay will be compared to bisulfite LINE1 pyrosequencing.

2. Materials and methods:

2.1. Clinical samples and cell lines

Cervical swabs were collected from routine Pap smears at the Department of Gynecological Outpatient, King Chulalongkorn Memorial Hospital, Chulalongkorn University, Bangkok and Khon Kaen University, Khon Kaen, Thailand. DNA was extracted from cervical cells by AllPrep DNA/RNA (QIAGEN) or total nucleic acid isolation kit (Roche). 280 samples were collected including normal (n=36), cervical intraepithelial neoplasia 1 (CIN1) (n=115), CIN2-3 (n=39) and squamous cell carcinoma (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. DNA extracted from normal white blood cells were used as normal control. This study has been approved (COA No. 087/2016) by the Institutional Review Board of the Faculty of Medicine, Chulalongkorn University.

2.2. 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 and SiHa) were used as cancerous cells control and DNA extracted from normal white blood cells were 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´- AAAATCAAAAAATTC CCTTTC-3' (150 bp). Sequencing primer and sequence to analyze were 5´-AGTTAGGTGTGGGATATAGT-3 and TTYGTGGTGYGTYGTTTTTTAAGTYGGTTT GAAAAGYGTA, respectively [19]. 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, Germany).

2.3 Quantification of global DNA methylation by ELISA-based assay

ELISA was performed according to instruction manual (5-mC DNA ELISA Kit, Zymoresearch)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, which was obtained from positive control DNA with known 5-methyl cytosine concentration, running on the same plate in duplicate.

2.4 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 CIN3+. P-value less than 0.05 was considered statistically significant.

3. Results

3.1. Methylation levels in cervical cancer cell lines

The optimal time for absorbance measurement is 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), 1.47 (C33A) and 8.20 of white blood cells collected from normal population. Bisulfite LINE1 pyrosequencing were 45% of Caski, 35% of SiHa and 62% of normal white blood cells.

3.2 Methylation levels in clinical samples

Mean of global DNA methylation by pyrosequencing in 161 cervical samples were as followed; 55.6% of normal, 56.8% of CIN1, 57.3% of CIN2-3 and 48.8% of cervical cancer, there was significant difference among groups ($p<0.05$) (Figure 1). By ELISA assay in 100 cervical samples, 4.50% of Normal, 3.83 of CIN1, 2.83% of CIN2-3 and 3.03% of cervical cancer, there was significant difference among groups ($p<0.05$) (Figure 2).

3.3 Sensitivity and specificity of ELISA and LINE1 pyrosequencing for diagnosis of CIN3+ lesions

We further analyzed whether the methylation level of a global DNA methylation by either bisulfite LINE1 pyrosequencing and ELISA could be used to predict lesion severity. Receiver operating characteristic (ROC) curve was used to calculate the sensitivity and specificity at different methylation percentages as a cut off point for diagnosis of CIN3+ lesions. Figure 3 and 4 showed ROC curve and area under curve (AUC) of LINE1 pyrosequencing and ELISA (AUC=0.72 and 0.60, respectively). At low cut off points (30%-45% of LINE1 pyrosequencing and 1.5%-2.0% of ELISA), there were high specificity but low sensivity as shown in Table 1 and 2.

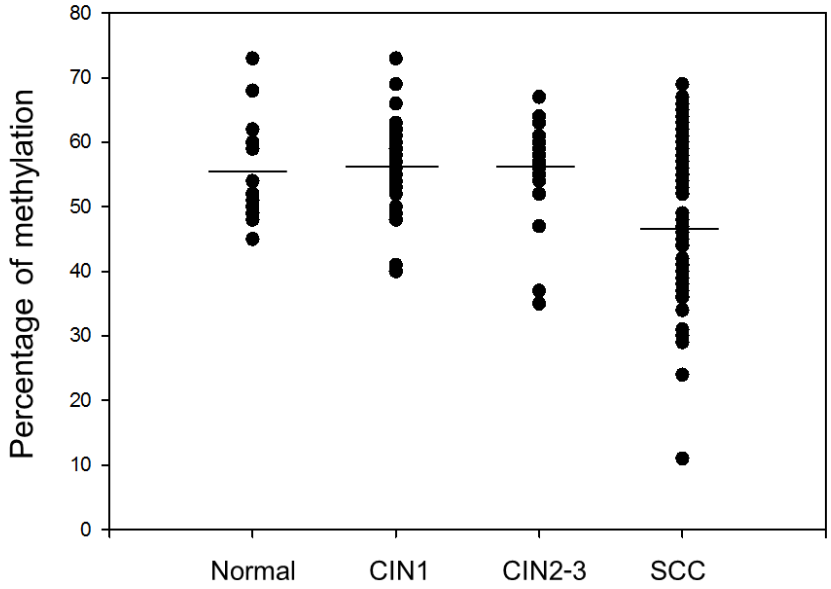

Figure 1. Methylation levels in 161 cervical samples with different lesions severity detected by bisulfite LINE1 pyrosequencing ($p<0.05$ (Kruskal wallis).

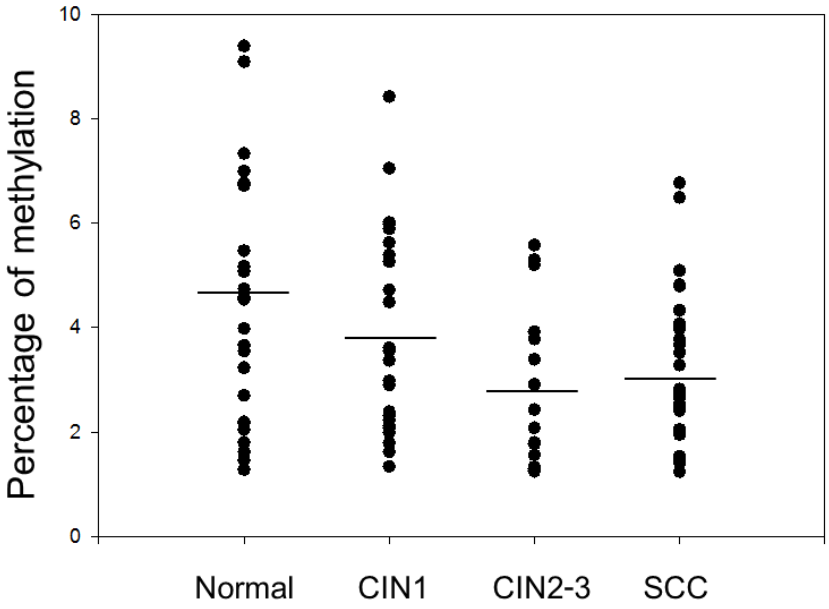

Figure 2. Methylation levels in 100 cervical samples with different lesions severity detected by ELISA assay ($p<0.05$ (Kruskal wallis).

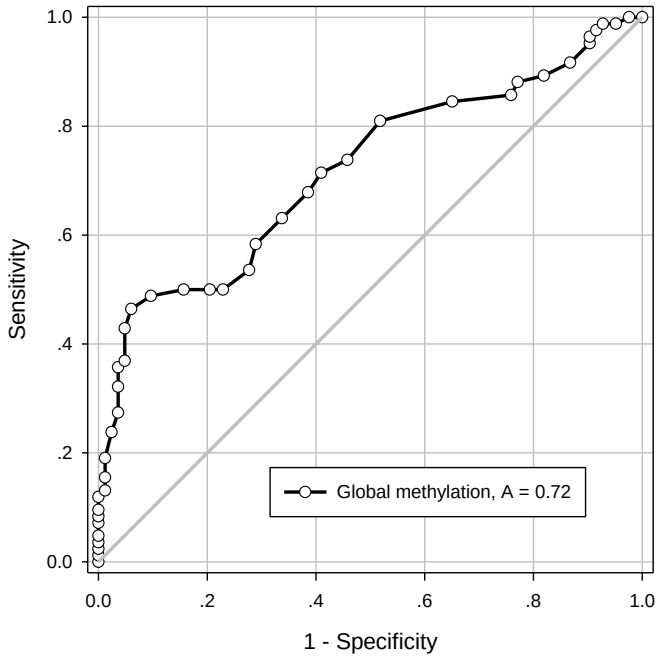

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Figure 3. Receiver operating characteristic (ROC) curve shows the sensitivity and specificity of methylation levels to diagnose cervical lesions as CIN3+ by bisulfite LINE1 pyrosequencing.

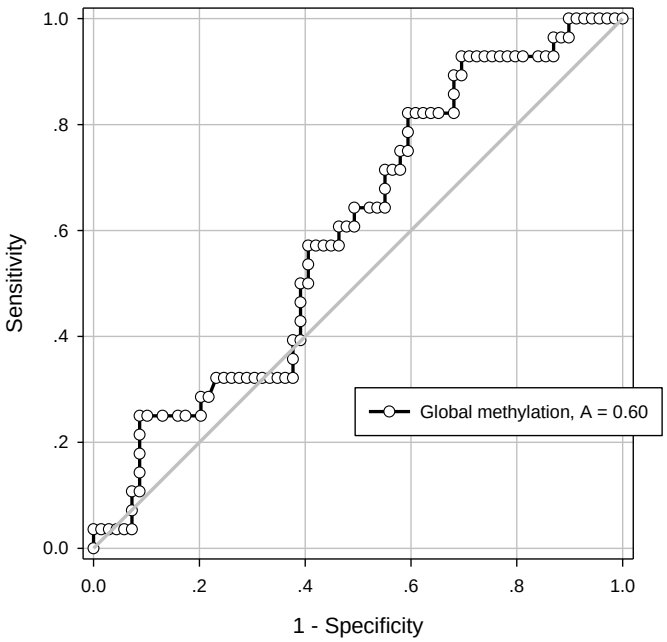

Figure 4. Receiver operating characteristic (ROC) curve shows the sensitivity and specificity of methylation levels to diagnose cervical lesions as CIN3+ by ELISA.

Table 1 Sensitivity and specificity of global DNA methylation to diagnose cervical lesions as CIN3+ by bisulfite 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

Table 2 Sensitivity and specificity of global DNA methylation to diagnose cervical lesions as CIN3+ by ELISA.

Cut off	1.5%		2.0%		2.5%		3.0%	
	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	3.5%		4.0%		4.5%		5.0%	
	Sensitivity	Specificity	Sensitivity	Specificity	Sensitivity	Specificity	Sensitivity	Specificity
%	64.29	50.72	75.0	42.03	82.14	39.13	89.29	31.88

4. Discussion

We found global DNA hypomethylation in cervical cancer samples compared to normal cervical cells that were in accordant to previous studies. Stacey N. 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%) [20]. Omid Fotouhi et al reported LINE1 hypomethylation in small intestinal neuroendocrine tumors (65%) compared to normal cells (75%) by pyrosequencing. By ELISA assay, mean of methylation index in cancer samples was 1.1 when compared to methylation index found in normal cells(1.2) [21]. Jean-Philippe Foy et al found hypomethylation in oral cancer compared non cancerous lesions [22]. LINE1 hypomethylation was found in various types of cancer such as bladder, colon, pancreas, prostate and stomach cancers [23]. The study of global methylation in cervical cancer was reported using high-performance capillary electrophoresis (HPCE) assay, they reported hypomethylation in cancer cells, normal, low grade lesions and high grade lesions were 2.81, 3.26, 3.37 and 3.22, respectively that suggested progressive hypomethylation [15].

However, due to low affinity of DNA binding to ELISA plate surface as seen by very low methylation percentage obtained from ELISA when compared to pyrosequencing assay. We will further investigate to increase the binding affinity of DNA to ELISA well strip and DNA elution buffer will be considered. Sensitivity can be increased by signal amplification reagent such as Tyramide signal amplification system that will be further studied.

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